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IMMUNOMODULATORY PROPERTIES OF GAMITHROMYCIN, DEXAMETHASONE AND KETOPROFEN IN LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION IN CALVES

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Immunomodulatory properties of gamithromycin, dexamethasone and ketoprofen in lipopolysaccharide-induced inflammation in calves

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APP	acute-phase protein
AUC	area under the curve
BW	body weight
C5a	complement factor 5a
CD14	cluster of differentiation 14
Cl	total body clearance
C_{max}	maximum plasma concentration
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
CV	coefficient of variation
DEX	dexamethasone
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
g	gravity
G	gauge
GAM	gamithromycin
Hp	haptoglobin
HPLC	high-performance liquid chromatography
HR	heart rate
IL	interleukin
IM	intramuscular
IP	intraperitoneal
IV	intravenous
k_{el}	elimination rate constant
KETO	ketoprofen
LBP	lipopolysaccharide binding protein
LOD	limit of detection
LOQ	limit of quantification

LOX	lipoxygenase
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
mCD14	membrane-associated CD14
MD-2	myeloid differentiation protein-2
MRM	multiple reaction monitoring
MRT	mean residence time
MS/MS	tandem mass spectrometry
MyD88	myeloid differentiation factor 88
m/z	mass-to-charge ratio
NE	no effect
NF-κB	nuclear factor-κB
NI	no increase
NO	nitric oxide
NR	not reported
NSAID	non-steroidal anti-inflammatory drug
p.a.	post administration
p.c.	post LPS challenge
PBMC	peripheral blood mononuclear cell
PD	pharmacodynamics
PG	prostaglandin
PGE_2-*met*	13,14-dihydro-15-keto PGA2
PIM	pulmonary intravascular macrophage
PK	pharmacokinetic
PLA_2	phospholipase A_2
ROA	route of administration
ROS	reactive oxygen species
RR	respiratory rate
RT	rectal body temperature
SAA	serum amyloid A
SAID	steroidal anti-inflammatory drug

SC	subcutaneous
sCD14	soluble CD14
SD	standard deviation
$t_{1/2el}$	half-life of elimination
TBC	time of blood collection
TLR4	Toll-like receptor 4
t_{max}	time to maximum plasma concentration
TNF	tumor necrosis factor
TOA	time of administration
TX	thromboxane
UPLC	ultra-performance liquid chromatography
UV	ultraviolet
V_d	volume of distribution

GENERAL INTRODUCTION

1. IMPORTANCE OF LIPOPOLYSACCHARIDE IN BOVINE VETERINARY MEDICINE

Lipopolysaccharide (LPS) is a structural part of the outer membrane of Gram-negative bacteria that is prerequisite for bacterial viability. The outer membrane acts as a protective barrier for the bacterium, with LPS contributing to this function. More specifically, phosphate diester bridges in the LPS-backbone are important in forming a cell surface structure, resistant to the penetration of harmful components such as penicillins (Tamaki *et al.*, 1971). In an animal host, LPS is not toxic when incorporated in the cell wall of the microorganism. However, during bacterial proliferation or cell death with subsequent lysis, LPS is released from the outer membrane. As a result, its toxic moiety, lipid A, induces an inflammatory response through activation of the innate immune system (Van Amersfoort *et al.*, 2003). From this point of view, the lipid A component of LPS is also referred to as endotoxin (Raetz and Whitfield, 2002). The innate immune system forms the first line of defence against microbial pathogens, with particularly blood monocytes, tissue macrophages and polymorphonuclear leukocytes contributing to this non-specific immune reaction (Miyake, 2004).

In bovine veterinary medicine, LPS is responsible for important clinical entities, including endotoxemia and Gram-negative sepsis (Cullor, 1992; Olson *et al.*, 1995). Endotoxemia refers to the presence of LPS in the circulation, whereas sepsis is defined as a combination of an infection and a systemic inflammatory response. With respect to sepsis, the infection source can be focal or generalized (Fecteau *et al.*, 2009). Particularly in calves, Gram-negative bacteria play a crucial role in the most frequent and economically important calf diseases, namely neonatal diarrhea and bovine respiratory disease (Michaels and Banks, 1988; Pardon *et al.*, 2012b). In this respect, *Escherichia coli*, *Mannheimia haemolytica* and *Pasteurella multocida* are the most frequently involved pathogens (Confer *et al.*, 1990; Constable, 2004; Harper *et al.*, 2011). Also in septicemia, which continues to be a life-threatening condition with high mortality risks – especially in calves with failure of passive transfer – the vast majority of isolates is Gram-negative (Fecteau *et al.*, 2009). The former pathologies are responsible for the majority of deaths in live-born neonatal calves < 1 month of age, which have been reported to have a mortality risk of 15-30% (Lofstedt *et al.*, 1999). Additionally, the prevalence and incidence risk for neonatal calf diarrhea has recently been

reported to be 19.1% and 21.2%, respectively (Bartels *et al.*, 2010; Windeyer *et al.*, 2014). The prevalence for bovine respiratory disease, on the other hand, depends on the production system, with particularly high incidences in white veal calves: 60% in the first three weeks after arrival at the farm (mean age at arrival: 17.4 ± 4.7 days) (Pardon *et al.*, 2012b; Pardon *et al.*, 2015). Also in cows, approximately 40% of the clinical cases of mastitis are caused by Gram-negative bacteria, including *E. coli*, *Klebsiella pneumoniae* and various species of *Enterobacter*. From those cows with severe Gram-negative mastitis, nearly 25% will either die or be culled due to complications (Bannerman *et al.*, 2003). Furthermore, LPS has been linked to infertility and subfertility, resulting from clinical or subclinical endometritis. This association can be attributed to the involvement of Gram-negative bacteria, including *E. coli*, in the pathogenesis of endometritis on the one hand, and to hormonal alterations induced by LPS on the other hand (Sheldon *et al.*, 2009). Endotoxin can also induce pathophysiologic responses, irrespectively of systemic bacterial infections. Accordingly, large amounts of LPS, derived from the Gram-negative bacterial flora, can enter the blood stream through the intestine when the latter is compromised by ischemia or inflammation. This translocation can subsequently induce endotoxemia (Werling *et al.*, 1996). Additionally, systemic exposure to LPS has been associated with the development of non-infectious diseases, including laminitis and displaced abomasum (Andersen, 2003).

The pathogenesis of Gram-negative bacterial infections is generally a fast process. In this respect, it should be remarked that, in contrast to bacterial exotoxins being toxic by killing host cells, the toxicity of LPS is mainly the consequence of the host's reaction to LPS (Rietschel *et al.*, 1994; Steiger *et al.*, 1999). If the animal does not overcome the infection early in the disease process, the outcome may not be altered substantially by treatments applied too late in the process (Cullor, 1992). The latter can be attributed to the fast development of the inflammatory response (within hours) on the one hand, and the late recognition by farmers on the other hand.

The former outlines the extensive involvement of this Gram-negative bacterial cell wall component in bovine veterinary medicine, and particularly in calves. In order to reduce morbidity and mortality, knowledge on the pathophysiologic changes induced by LPS is imperative.

2. LIPOPOLYSACCHARIDE SIGNALING

2.1 CHEMICAL STRUCTURE OF LIPOPOLYSACCHARIDE

Gram-negative bacteria typically contain a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane (Fig. 1). LPS is the dominating component of this outer membrane, consisting of a hydrophilic heteropolysaccharide and the covalently bound toxic lipid A moiety (Rietschel *et al.*, 1993) (Fig. 2). With respect to *Enterobacteriaceae* such as *E. coli*, the heteropolysaccharide component comprises two regions which differ in their genetic determination, biosynthetic pathways and chemical structure: the O-antigen and the core region. Additionally, the core segment can be formally subdivided into an outer and an inner portion (Rietschel *et al.*, 1994). The lipid A domain anchors the LPS molecule to the outer membrane, whereas the O-antigen is exposed on the outer surface of the bacterium (Trent *et al.*, 2006).

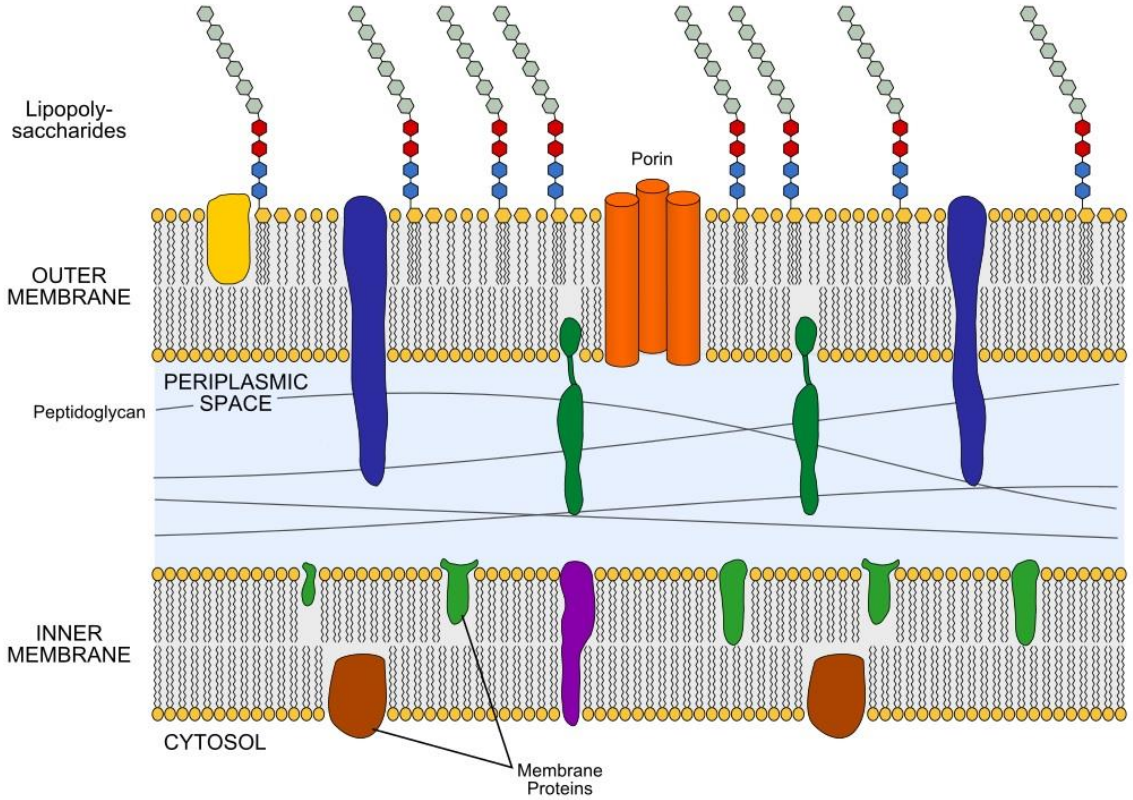

Figure 1. Gram negative cell wall structure (adapted from Dahl, 2008)

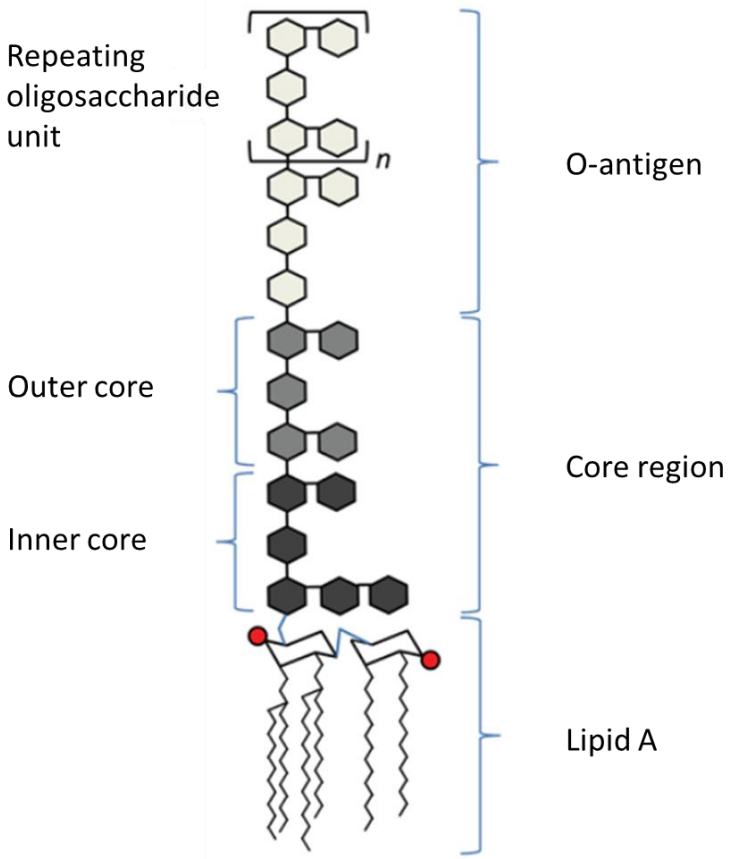

Figure 2. Molecular structure of the LPS molecule
(adapted from Maeshima and Fernandez, 2013)

Among different bacterial strains, the LPS molecule exhibits a large variability, which can be attributed to the composition of the core region and the O-antigen. Particularly the latter shows a remarkable structural diversity, as more than 60 monosaccharides and 30 different noncarbohydrate components have been recognized (Raetz and Whitfield, 2002). The lipid A moiety, on the other hand, is a highly conserved pattern among Gram-negative bacteria. In this respect, the host's LPS receptors are targeted against the lipid A domain, whereas the O-antigen on the surface defines the serological specificity of the bacterium (Rietschel *et al.*, 1994; Jerala, 2007).

2.2. INNATE RECOGNITION OF LIPOPOLYSACCHARIDE

The release of LPS from the bacterial surface results in the activation of the innate immune response, including blood monocytes, tissue macrophages and polymorphonuclear leukocytes (Miyake, 2004). The receptor that is responsible for delivering an LPS signal and that is expressed on the surface of these innate immune cells, has been identified as Toll-like receptor 4 (TLR4). In this context, three cooperating extracellular molecules are involved in the extracellular recognition of LPS: LPS-binding protein (LBP), cluster of differentiation 14 (CD14) and myeloid differentiation protein (MD)-2. These proteins chaperone the LPS-molecule from the bacterial membrane to the transmembranar TLR4 (Jerala, 2007) (Fig. 3). Briefly, the LBP first binds to the lipid A moiety of LPS. LBP then catalyses the transfer to either membrane-associated CD14 (mCD14), which is present on cells of the innate immune system, or to circulating soluble CD14 (sCD14). Subsequently, CD14 translocates LPS to MD-2, an extracellular molecule that is associated with TLR4. The latter binding leads to the recruitment of adaptor proteins to the intracellular domain of TLR4, in this way triggering the intracellular signaling cascade (Miyake, 2004; Peri *et al.*, 2010).

2.3. INTRACELLULAR SIGNALING PATHWAY

The intracellular signaling ultimately results in the activation of nuclear factor-κB (NF-κB). NF-κB is the generic name of a family of transcription factors that regulate the expression of a large number of genes involved in immune and inflammatory processes. NF-κB transcription factors are activated in response to various stimuli, including LPS. In this respect, two pathways are involved in TLR4 signaling: an early myeloid differentiation factor 88 (MyD88)-dependent (Fig. 3) and a delayed MyD88-independent pathway. The first was shown to be responsible for pro-inflammatory cytokine expression, whereas the latter is rather linked to the induction of type I interferons (Lu *et al.*, 2008; Verstrepen *et al.*, 2008). Overall, these inflammatory mediators activate an assortment of inflammatory cascades, intending to control the Gram-negative bacterial infection. This predetermined and well-orchestrated sequence of processes is referred to as the acute-phase response (Baumann and Gauldie, 1994; Wyns *et al.*, 2015b).

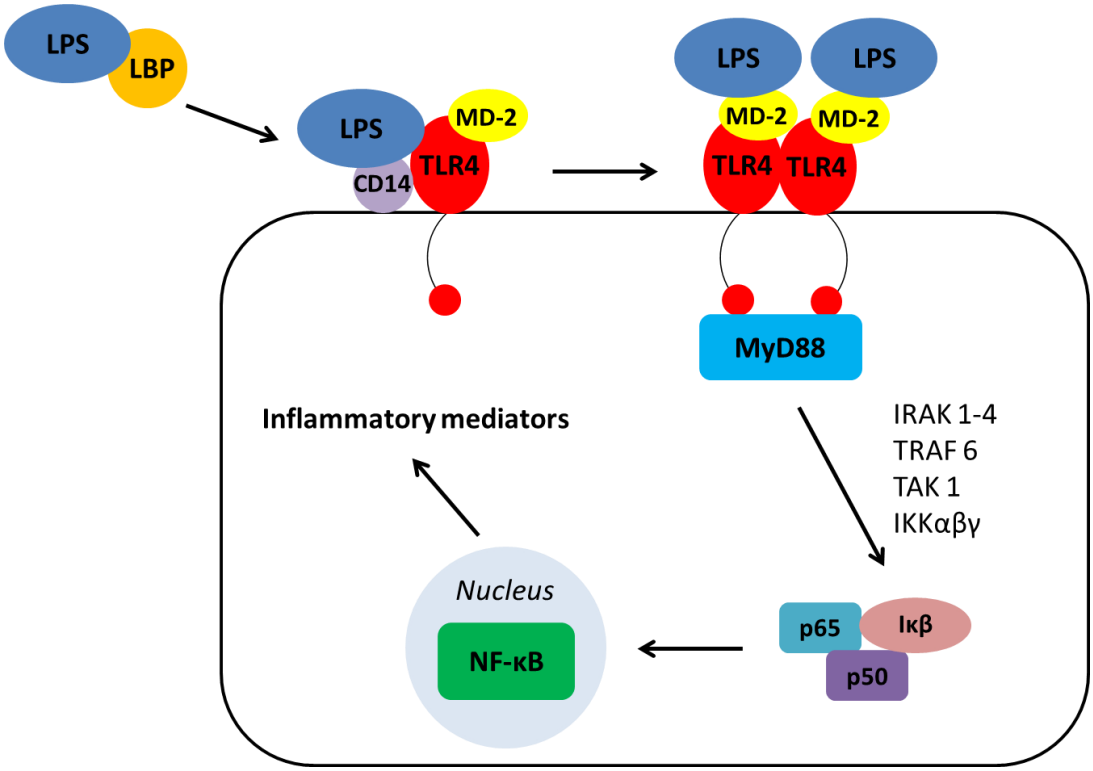

Figure 3. Schematic overview of MyD88-dependent TLR4 activation (adapted from Brookes *et al.*, 2009)

3. LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION IN CATTLE

The intravenous (IV) administration of purified standard reference LPS has been shown to induce an acute systemic inflammatory response, which in part corresponds to Gram-negative sepsis. In this respect, the suitability of experimental endotoxemia models to study the pathophysiology of sepsis and septic shock has been suggested decades ago (Hoffman and Natanson, 1993; Remick and Ward, 2005; Andreasen *et al.*, 2008). These models indeed provide several advantages regarding standardization and reproducibility. Nevertheless, counter-arguments are being reported as well, due to the lack of an infectious focus and a sepsis-induced immune reaction following an IV LPS challenge (Redl *et al.*, 1993; Olson *et al.*, 1995; Remick and Ward, 2005). Despite this questioning, endotoxemia models have been confirmed to represent an adequate tool for understanding inflammatory processes and correlated non-specific symptoms (Poli-de-Figueiredo *et al.*, 2008).

Following an IV endotoxin challenge in cattle, LPS is cleared from the plasma within 15 minutes (van Deventer *et al.*, 1990; Redl *et al.*, 1993; Ohtsuka *et al.*, 1997a). As a result of the rapid response of the innate immune system, pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6, and other inflammatory mediators, including prostaglandins (PGs) are released from cells of the monocytic lineage. These mediators subsequently trigger the acute-phase response, which is characterized by leukocyte activation, mobilization of phagocytes, altered plasma concentrations of zinc, iron, calcium and copper and the synthesis of acute-phase proteins by hepatocytes. Major acute-phase proteins in cattle are serum amyloid A (SAA) and haptoglobin (Hp). Clinically, on the other hand, the acute-phase response is recognized by fever, tachycardia and behavioural changes such as depression, anorexia and hyperalgesia (Canning and Baker, 1990; Alsemgeest *et al.*, 1994; Watkins *et al.*, 1994; Van Miert, 1995; Paape *et al.*, 2002; Borderas *et al.*, 2008; Eckersall and Bell, 2010; Zebeli *et al.*, 2010). In general, the acute-phase response is regarded to be beneficial, as it is important for providing protection to the animal, as well as to return the organism to normal function (Baumann and Gauldie, 1994; Carroll *et al.*, 2009b). Nevertheless, when the reaction becomes dysregulated or excessive, it can result in life-threatening syndromes, such as sepsis and septic shock (Lillie, 1974; Peri *et al.*, 2010).

Cattle, and in particular calves, have been shown to be extremely sensitive to LPS in comparison with other animal species (Michaels and Banks, 1988). In this respect, a study of Berczi *et al.* (1966) revealed a lethal dose of 0.025 mg/kg body weight (BW) of LPS (O78 *E. coli* strain) for calves following intraperitoneal (IP) administration, whereas for rabbits, dogs, swine and chickens, lethal doses of 3.0, 4.0, 5.0 and 50 mg/kg BW, respectively, were reported. Humans, on the other hand, are even more sensitive to endotoxin as a dose of 2-4 ng/kg BW can be used to study the pathophysiology of sepsis (Suffredini *et al.*, 1989). Additionally, doses as low as 0.06-0.2 ng/kg have been demonstrated to induce low-grade inflammation in humans (Starkie *et al.*, 2003).

3.1. *IN VIVO* BOVINE LIPOPOLYSACCHARIDE INFLAMMATION MODELS

In order to study the bovine acute-phase response, various experimental designs have been reported in cattle. Overall, *E. coli* LPS is the most frequently applied type of endotoxin with respect to the induction of an inflammatory response. Table 1 provides a chronological overview of bovine *E. coli* LPS inflammation models, which were used to study the responses of cytokines and/or acute-phase proteins. As can be observed from this table, the IV administration of endotoxin is clearly the most common route of administration. Additionally, the selected *E. coli* serotype, dose, duration of administration and the techniques used for the determination of inflammatory mediators are included. Both the O55:B5 and O111:B4 serotypes are frequently applied in 61 and 33% of the studies, respectively. Particularly the earlier studies used the O55:B5 serotype, whereas the O111:B4 serotype was selected more frequently in recent experiments. This evolution might be correlated with the not strictly reproducible inflammatory response following challenges using the O55:B5 serotype. Doses of LPS range from 0.01 to 25 µg/kg BW and 0.1 to 2.5 µg/kg BW for the O55:B5 and O111:B4 serotype, respectively. Regarding the duration of administration, single LPS bolus doses and continuous infusions can be distinguished. Although it seems plausible that an infusion would mimic a clinical endotoxemia more closely, Carroll *et al.* (2009b) reported that this difference is rather theoretical than practical (Olson *et al.*, 1995). This can be concluded from the study of Gerros *et al.* (1993) as well, since both an IV LPS bolus and an IV infusion resulted in the same sequence of mediator

release. The latter authors revealed that the duration and magnitude of the inflammatory response, on the other hand, can be related to the dose of endotoxin. Earlier studies generally used bioassays for analysis of mediators, whereas enzyme-linked immunosorbent assays (ELISAs) are currently regarded to be the gold standard (Elshal and McCoy, 2006). In this respect, ELISAs measure both free and receptor-bound cytokines, while bioassays only determine biologically active cytokines (Myers *et al.*, 1999). Based on the advantages of multiplex flow cytometry assays, such techniques for the simultaneous detection of multiple inflammatory mediators are gaining popularity. More specifically, these techniques reduce the required sample volume and analysis time, and concurrently provide a wider dynamic range (Wyns *et al.*, 2013).

Regarding the research of Gram-negative mastitis in cows, the intramammary injection of *E. coli* LPS is a frequently applied method (Bannerman *et al.*, 2003; Lehtolainen *et al.*, 2004; Vels *et al.*, 2009). However, as this type of experiments diverges too much from systemic IV LPS challenges and were not the subject of this doctoral thesis, these studies were not included in Table 1.

With respect to other types of LPS than *E. coli*, information is much more limited. The IV administration of endotoxin from a field isolate of *P. multocida* B:2 has been studied in buffalo calves by Horadagoda *et al.* (2002). This challenge resulted in peak values of TNF-α at 1-2 h post LPS challenge (p.c.), as well as an increase of Hp concentrations. Additionally, Kwon *et al.* (2011) reported increased levels of Hp following the subcutaneous (SC) administration of *Salmonella typhimurium* LPS in 56-days-old calves.

Table 1. *In vivo Escherichia coli* LPS inflammation models in calves with respect to cytokines and acute-phase proteins.

LPS		Animals			Time to maximal cytokine levels (h p.c.)			Acute-phase proteins (APP)		Technique for cytokine/APP analysis	Reference
Serotype	Dose (µg/kg BW) (duration of infusion)	N°	Age (d)	BW (kg)	TNF-α	IL-1β	IL-6	SAA	Hp		
Intravenous LPS administration											
O55:B5	1.5 (0.5 h) 0.5 (2.5 h)	4	2-4	40.4-48.6	1-2	-	-	-	-	Bioassay	Adams *et al.*, 1990
O55:B5	0.5	2	14-21	45-60	1-2	-	-	-	-	Radioimmunoassay	Peel *et al.*, 1990
O55:B5	1.0	4*	100	90	2	-	-	-	-	Radioimmunoassay	Kenison *et al.*, 1991
O55:B5	Bolus: 0.2; 2.0 or 20 Infusion (0.83 h): 0.2; 2.0 or 20	25*	1	43.0 ± 6.1	1-1.5	3-3.5	-	-	-	Bioassay	Gerros *et al.*, 1993
O55:B5	1.5 µg/kg/h (0.5 h) 1 µg/kg/h (2.5 h)	6*	1	32.7-53	2	-	-	-	-	Bioassay	Semrad *et al.*, 1993
O55:B5	5 (0.17 h)	6*	7-8	46.5	1	-	-	-	-	Radioimmunoassay	Kinsbergen *et al.*, 1994
O111:B4	2 (1.67 h)	6*	-	346 ± 19	0.4-0.8	Biphasic: 0.67 / 5	Biphasic: 2 / 5.5	Increase	NI	Bioassay/SAA: ELISA; Hp: Hb binding assay	Werling *et al.*, 1996
O55:B5	25	5*	-	60-180	1	-	-	-	-	Bioassay	Ohtsuka *et al.*, 1997a
O26:B6	25	7*	-	550-800	2	2	-	-	-	Bioassay	Ohtsuka *et al.*, 1997b
O111:B4	0.1	8	22 ± 2	46.7 ± 6.3	2	-	-	-	-	Bioassay	Bieniek *et al.*, 1998
O111:B4	2 (1.67 h)	6*	-	311 ± 5.1	1.5-2	-	-	-	-	Bioassay	Steiger *et al.*, 1999
O55:B5	0.01; 0.1; 1	8	-	603 ± 59	-	-	-	Increase	Increase	ELISA	Jacobsen *et al.*, 2004
O55:B5	0.2	12	120-150	-	1	-	-	Increase	Increase	Radioimmunoassay/ ELISA	Elsasser *et al.*, 2005
O55:B5	0.25	8	-	328 ± 6	1	-	-	Increase	Increase	Radioimmunoassay/ ELISA	Kahl and Elsasser, 2006
O55:B5	0.05	30	14	-	2	-	-	-	Increase	ELISA/ELISA	Kushibiki *et al.*, 2008
O111:B4	1	18	-	233 ± 5	1.5	4.5-5	4-4.5	-	NI	ELISA/Hb binding assay	Carroll *et al.*, 2009a
O111:B4	2.5	9	-	299 ± 5	1.4	3.0	4.3	Increase	-	ELISA/ELISA	Carroll *et al.*, 2009b
O111:B4	0.5	21	-	310	1.5	-	3.5-4	-	-	ELISA	Carroll *et al.*, 2013
Subcutaneous LPS administration											
O111:B4	4	42	9 ± 1	-	-	-	-	-	Increase	Hb binding assay	Ballou, 2012

BW: body weight; d: days; h p.c.: hours post LPS challenge; SAA: serum amyloid A; Hp: haptoglobin; Hb: haemoglobin; NI: no increase; -: not investigated; * studies including negative control calves (saline)

3.2. LIPOPOLYSACCHARIDE-INDUCED ACUTE-PHASE RESPONSE

3.2.1. Inflammatory mediators

As mentioned before, the acute-phase response is initiated by the release of several inflammatory mediators. Firstly, cytokines are characterized by functional pleiotropy and redundancy, which complicates the understanding of their hierarchal actions (Baumann and Gauldie, 1994). TNF-α has been put forward to be the principal mediator of acute inflammation in response to Gram-negative bacteria (Conti *et al.*, 2004). As a result, this cytokine is the most frequently studied inflammatory mediator following endotoxin challenges. Table 1 demonstrates that this is also the case in cattle in *in vivo E. coli* LPS inflammation models. Peak levels of TNF-α were determined in all studies before 2 h p.c. Regarding the two related proteins IL-1α and IL-1β, only changes of IL-1β were reported. Nevertheless, analysis of IL-1β and IL-6 was performed less frequently, and maximal levels of these cytokines were recorded 1-4 h after the peak of TNF-α in all experiments. It should be mentioned, however, that different analytical techniques for cytokine determination may yield different results (bioassays/ELISAs).

In addition to cytokines, eicosanoids such as prostaglandins, thromboxanes (TXs) and leukotrienes contribute to the acute-phase response. These lipid mediators are synthetized *de novo* from membrane phospholipids in response to trauma or inflammatory stimuli like LPS (Fig. 4). In this respect, a cascade of enzymes is involved, with phospholipase A_2 (PLA_2) coordinating the initial step: the release of arachidonic acid from membrane phospholipids (Henderson, 1994). Moreover, the upregulation of TLR4 has been demonstrated to lead to an excessive production of PLA_2 (Jiang *et al.*, 2003). Released arachidonic acid is the common precursor molecule of all eicosanoids, and is subsequently converted to prostaglandins and thromboxanes by the cyclooxygenase (COX) pathway and to leukotrienes by the 5-lipoxygenase (LOX) pathway (Henderson, 1994).

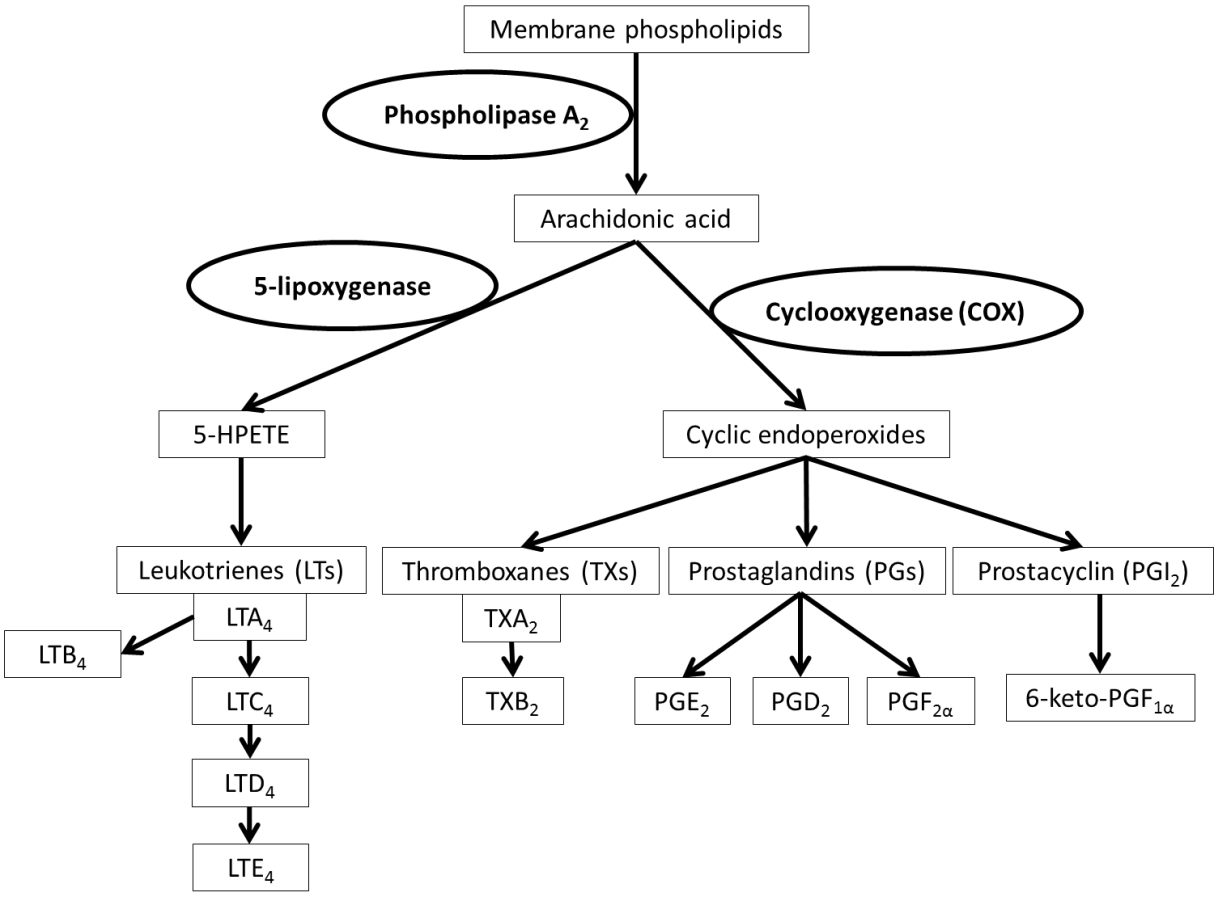

Figure 4. The arachidonic acid pathway (based on Adams, 2001)

All mentioned pro-inflammatory cytokines contribute to the induction of the hepatic synthesis of acute-phase proteins. Still, IL-6 has been reported to be the major mediator, in cooperation with glucocorticoids (Le and Vilcek, 1989; Gabay and Kushner, 1999; Petersen *et al.*, 2004). Several review articles have gathered the current knowledge on these inflammatory parameters in cattle (Petersen *et al.*, 2004; Gruys *et al.*, 2005; Eckersall and Bell, 2010; Ceciliani *et al.*, 2012). The general functions of acute-phase proteins include the elimination of pathogenic agents, tissue repair and restoring homeostasis (Ceciliani *et al.*, 2002). Depending on their respective increased or decreased plasma concentrations following an inflammatory response, acute-phase proteins are divided in positive and negative acute-phase proteins (Gabay and Kushner, 1999). As mentioned before, the major positive bovine acute-phase proteins are SAA and Hp (Ceciliani *et al.*, 2012). Plasma concentrations of these proteins generally increase 10-100 fold during an acute phase response (Cray *et al.*, 2009). The biological functions of SAA are far from elucidated. The

currently confirmed functions of this acute-phase protein include the transport of cholesterol from dying cells to hepatocytes and mediation of the migration, adhesion and tissue infiltration of monocytes and neutrophils (Badolato *et al.*, 1994; Manley *et al.*, 2006). Furthermore, SAA is an innate immune opsonin for Gram-negative bacteria and it has anti-inflammatory properties, for instance through inhibition of the respiratory burst of leukocytes (Linke *et al.*, 1991; Shah *et al.*, 2006). The primary function of Hp covers the prevention of loss of iron through the formation of very stable complexes with free haemoglobin in the blood. In this way, Hp exerts a bacteriostatic effect by restricting the availability of iron for growing bacteria (Petersen *et al.*, 2004). Similarly to SAA, Hp possesses anti-inflammatory capabilities, such as the inhibition of neutrophil respiratory burst activity, granulocyte chemotaxis and phagocytosis (Rossbacher *et al.*, 1999). In contrast to the determination of pro-inflammatory cytokines following the IV administration of LPS in cattle, acute-phase proteins were not included in the majority of papers (Table 1). Nevertheless, both SAA and Hp plasma concentrations have been reported to increase. SAA levels increased significantly in all involved studies, whereas Werling *et al.* (1996) and Carroll *et al.* (2009a) detected no alterations in Hp levels. The latter may be explained by the limited sampling period (up to 6 and 8 h p.c., respectively), while maximal levels of acute-phase proteins are typically reached 24-48 h p.c. (Jacobsen *et al.*, 2004; Kushibiki *et al.*, 2008). Moderate positive acute phase proteins in cattle (≤ 10 fold increase) are α1-acid glycoprotein, LBP and fibrinogen (Cray *et al.*, 2009; Ceciliani *et al.*, 2012). Due to their rather subtle increases during an inflammatory response, it is advisable to combine the results of these moderate acute phase proteins with other parameters, including other (major) acute phase proteins and total leukocyte counts, in order to optimize the accuracy of the diagnosis (Ganheim *et al.*, 2007).

In response to the release of inflammatory mediators, a well-orchestrated series of clincial reactions is initiated (Fig. 5). In the following sections, these acute-phase response-related clinical signs in cattle are illustrated more into detail: the pulmonary response, behavioural changes, fever and tachycardia. Additionally, the blood biochemical responses will be discussed briefly.

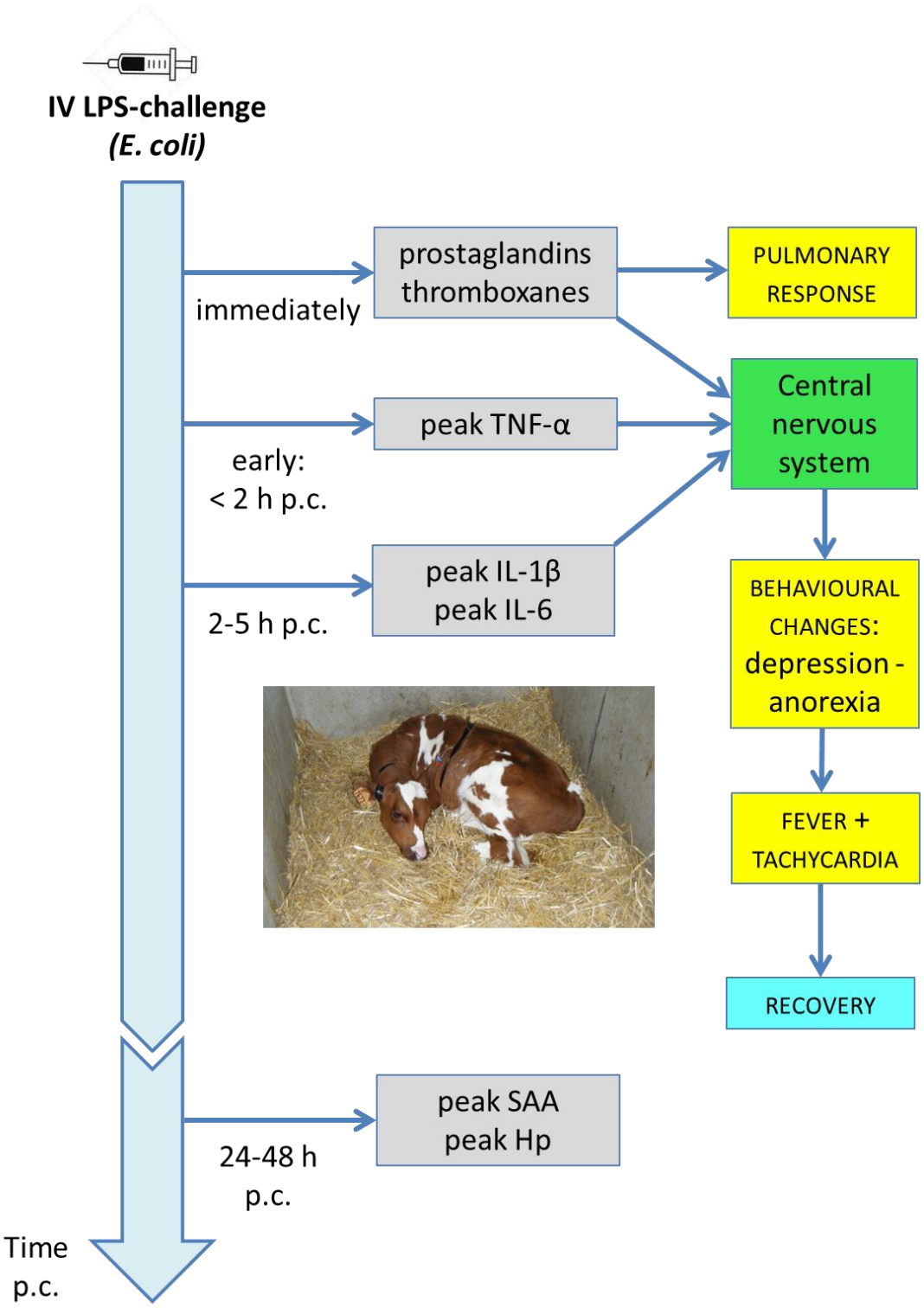

Figure 5. Overview of the release of inflammatory mediators and the induced clinical signs following an intravenous *E. coli* LPS challenge

3.2.2. Clinical signs

3.2.2.1. Pulmonary response

The lung has been described to be the target organ for endotoxin in cattle (Tikoff *et al.*, 1966). This finding can be related to the first clinical sign following an IV LPS challenge, being the occurrence of severe pulmonary distress. The latter is characterized by dyspnea, coughing and abnormal breathing sounds like stridor and stertor. These symptoms generally occur within 30 min p.c. (Kenison *et al.*, 1991; Bieniek *et al.*, 1998; Jacobsen *et al.*, 2004). Nevertheless, higher doses of LPS seem to result in a faster appearance of respiratory distress (Tikoff *et al.*, 1966; Ohtsuka *et al.*, 1997b). In this respect, a direct toxic effect of LPS to bovine pulmonary endothelial cells can be hypothesized. Such an effect was previously confirmed for *M. haemolytica* endotoxin in an *in vitro* study (Paulsen *et al.*, 1989). Additionally, pulmonary intravascular macrophages (PIMs), which are constitutively present in cattle, have been reported to be responsible for eliminating blood-borne toxins, including endotoxin (Winkler, 1988). The clearance of LPS by PIMs, however, results in the excessive release of eicosanoids. These mediators, and particularly TXA_2, are regarded to play a role in the early hemodynamic effects of endotoxin, including a sharp rise in pulmonary arterial pressure (Tikoff *et al.*, 1966; Olson and Brown, 1986). They are also involved in the increased pulmonary vascular resistance and decreased dynamic lung compliance, which coincide with the rise in pulmonary arterial pressure and contribute to the development of dyspnea (Esbenshade *et al.*, 1982; Olson and Brown, 1986). Indeed, the presence of LPS in cytoplasm of PIMs was confirmed as soon as 8-10 min after the IV administration of 1 µg/kg BW *E. coli* LPS in sheep, with simultaneous development of pulmonary interstitial oedema (Singh and Atwal, 1997).

3.2.2.2. Behavioural changes

Following an LPS challenge, general depression and anorexia can be recognized in cattle (Borderas *et al.*, 2008). Particularly IL-1β and TNF-α have been put forward with respect to these behavioural changes, due to depression of the central nervous system. As these

peripherally released cytokines cannot pass the blood-brain barrier passively, their actions on the brain involve two pathways: a fast neural transmission pathway through afferent nerves from the site of inflammation and a slower humoral transmission pathway which involves cytokine production in the choroid plexus and circumventricular organs, where the blood-brain barrier is deficient. Brain cytokines subsequently diffuse into the brain parenchyma by volume transmission, activating neurons and neural pathways relevant for expression of sickness behaviour (Konsman *et al.*, 1999; Johnson, 2002; Dantzer, 2009). In both pathways, these cytokine actions can be mediated by prostaglandins, which are synthetized by endothelial cells of brain venules in response to circulating cytokines (Engblom *et al.*, 2002).

The systemic or central administration of IL-1β or TNF-α to rats and mice has been shown to induce the full spectrum of behavioural signs of sickness (depressed locomotor activity, decreased exploration of the physical and social environment, reduced food and water intake and impaired learning and memory), whereas IL-6 exerts no behavioural effects (Dantzer, 2001). Nevertheless, IL-6 potentiates the behaviourally depressing effects of IL-1β (Lenczowski *et al.*, 1999). Particularly brain IL-1β has been reported to be potent at reducing appetite in mice (Layé *et al.*, 2000). Most bovine studies indeed report a decrease in food intake following experimental endotoxemia, although it has been reported to be a dose-dependent and short-lived effect (Kenison *et al.*, 1991; Johnson and von Borell, 1994; Elander *et al.*, 2007).

The role of PGE_2 in behavioural depression and anorexia has been confirmed repeatedly as well (Pecchi *et al.*, 2009). Teeling *et al.* (2007) even demonstrated that low grade inflammation impacts on the brain independently of peripheral cytokine production, in this way suggesting a key role for prostaglandins. However, sub-pyrogenic doses of LPS were used in this murine study, and the effect on non-classic sickness behaviour was studied, including exploration and anxiety behaviour.

3.2.2.3. Fever

The onset of fever is one of the most frequently studied effects following an endotoxin challenge. In cattle, maximal body temperatures are generally reached around 4-5 h p.c. (Kinsbergen *et al.*, 1994; Steiger *et al.*, 1999; Borderas *et al.*, 2008; Carroll *et al.*, 2009b). Fever is beneficial up to a certain point as it inhibits the growth of certain microorganisms and increases the rate of enzyme reactions. As a result, the body's metabolism enhances, with stimulation of phagocytosis, immune responses, and tissue repair (Ceciliani *et al.*, 2002). In the course of time, the knowledge on fever development has evolved considerably. The "conventional view" states that the febrile response is initiated by the peripheral production of pyrogenic cytokines, TNF-α, IL-1β and IL-6 being the most relevant. In fact, IL-6 appears to be the most prominent cytokine with respect to mediating fever (Conti *et al.*, 2004). As mentioned before, PGE_2 synthesis in the brain will be induced next, where this lipid mediator will act on the thermoregulatory centre in the preoptic area of the anterior hypothalamus (Blatteis *et al.*, 2005). As a result, the thermoregulatory set point will increase, leading simultaneously to increased heat production and decreased heat loss (Johnson, 2002). This view was confirmed in calves as the IV administration of recombinant bovine TNF-α resulted in dose-dependent changes in the rectal body temperature (Kushibiki *et al.*, 2000). Also in adult cattle, recombinant bovine IL-1β induced the onset of fever, while the effect of recombinant bovine IL-6 has not been studied in this animal species (Goff *et al.*, 1992; Godson *et al.*, 1995). The more recent insights in the course of fever development in humans and laboratory animals, on the other hand, emphasise that the onset of the febrile response precedes the systemic appearance of pyrogenic cytokines (Blatteis, 2007). Indeed, cytokines are not constitutively expressed in mononuclear phagocytes, and their *de novo* synthesis in response to pyrogenic stimuli requires some time, resulting in a more delayed PGE_2 synthesis in the brain (Conti *et al.*, 2004). Moreover, Steiner *et al.* (2006) demonstrated that circulating PGE_2, released from macrophages within LPS-processing organs like the lung and liver, initiated fever in the early phase (0.5 h) of infection in rats. Similarly to PIMs, Kupffer cells in the liver contribute to the clearance of circulating endotoxin, yet to a larger extent in comparison with cattle since these are the primary LPS clearing cells in humans and laboratory animals (Blatteis, 2007). Besides the subsequent production of cytokines, the

transcription of inflammation-induced COX-2 is upregulated in Kupffer cells, leading to the activation of peripheral PGE_2 synthesis (Li *et al.*, 2006). The need for COX-2 transcription, however, might again lag the onset of the febrile response (Blatteis *et al.*, 2005). Consequently, the immediate activation of the complement cascade by LPS has been hypothesized to be an important trigger of COX-1-induced PGE_2 formation. This pathway implies the production of anaphylatoxin C5a, and its subsequent binding to its receptor on Kupffer cells, resulting in an early peripheral fever trigger through excitation of hepatic vagal afferents (Blatteis *et al.*, 2005; Blatteis, 2007). In this respect, a similar pathway might be of importance in cattle, both in the liver and the lung. The ability of PGE_2 to directly pass from the blood to the brain, on the other hand, has been questioned increasingly (Morimoto *et al.*, 1992; Blatteis *et al.*, 2005).

3.2.2.4. Tachycardia

Kinsbergen *et al.* (1994) and Bieniek *et al.* (1998) reported nearly coinciding maxima for body temperature and heart rate following an LPS challenge in calves. In this respect, it has been suggested that fever exerts a negative inotropic effect, with an increased heart rate compensating for the decreased contractility and the increased peripheral oxygen demand (Haupt and Rackow, 1983). Moreover, both central nervous system-mediated effects and cardiac-mediated mechanisms have been put forward with reference to the tachycardic effects of cytokines during fever and inflammation (Takayama *et al.*, 2005; Nalivaiko, 2006).

3.2.2.5. Blood biochemical responses

An endotoxin challenge has been demonstrated to alter plasma concentrations of zinc, iron, calcium and copper (van Miert, 1995; Zebeli *et al.*, 2010). Plasma zinc and iron levels decrease in response to the release of pro-inflammatory cytokines. More specifically, these cytokines induce the synthesis of metallothionein, which enhances the intracellular metal ion binding capacity (Gruys *et al.*, 2005). As described for Hp, lower iron concentrations are beneficial to the host, as iron is essential for microbial growth. Additionally, decreased serum zinc values are beneficial during an inflammatory response, since increased zinc levels

have been shown to impair phagocytic functions (Sugarman, 1983). Regarding the decreased levels of calcium following LPS administration, on the other hand, it remains unclear whether this is associated with the detoxification of endotoxin or with impaired calcium mobilization (Zaloga *et al.*, 1992; Garidel *et al.*, 2005; Zebeli *et al.*, 2010). In contrast to the lower plasma concentrations of zinc, iron and calcium during an inflammatory condition, plasma levels of copper increase, enhancing the host's antimicrobial responses (Hodgkinson and Petris, 2012). These elevated levels can be partially attributed to increased serum concentrations of the copper-binding protein, ceruloplasmin (Erskine and Bartlett, 1993).

3.3. COMPARISON WITH BACTERIAL INFECTIONS IN CATTLE

In addition to endotoxin challenges, experimental infections with Gram-negative bacteria have been frequently applied in cattle in view of the study of the acute-phase response. In this respect, experiments using *E. coli*, *S. typhimurium*, *P. multocida*, *M. haemolytica* and *Histophilus somni* have been performed, based on the similarity with natural occurring diseases in cattle, including mastitis, enteritis and bovine respiratory disease. Due to multifactoriality, these experimental bacterial infections generally result in higher inter-animal variations compared with the administration of LPS, both with respect to fever development and plasma concentrations of inflammatory mediators (Horadagoda *et al.*, 2002).

Regarding the profile of pro-inflammatory cytokines following non-intramammary experimental bacterial infections in cattle, data are limited to TNF-α. More specifically, elevated plasma concentrations of this cytokine were only demonstrated in two studies, applying an intra-tracheal inoculation with *M. haemolytica* (Pace *et al.*, 1993; Horadagoda *et al.*, 1994). The inoculum used by Horadagoda *et al.* (1994) contained a higher number of colony forming units than the inoculum of Pace *et al.* (1993) (4×10^{10} and 3.5×10^{7}, respectively). In this respect, the first study reported maximal levels of TNF-α already 2 h after inoculation, whereas in the second study, peak concentrations were measured at 8 h post inoculation. The oral inoculation of calves with *S. typhimurium*, on the other hand, did

not result in increased levels of TNF-α (Peel *et al.*, 1990). It should be mentioned, however, that blood collection in this study was limited to a once-daily sampling.

In contrast with the assessment of cytokine profiles, acute-phase proteins have been evaluated more frequently following experimental infections. The underlying idea here is the possibility to use acute-phase proteins as biomarkers of infection and inflammation (Eckersall and Bell, 2010). Additionally, these inflammatory mediators show a more prolonged presence in plasma compared to cytokines. Table 2 provides a brief overview of the published studies investigating acute-phase proteins in calves in experimental models of bacterial respiratory infections.

Table 2. Experimental models exploring the effect of a bacterial respiratory infection on serum amyloid A (SAA) and haptoglobin (Hp) in calves.

Bacterium	SAA	Hp	Reference
Mannheimia haemolytica	-	Increase	Conner *et al.*, 1989
	Increase	Slow increase	Horadagoda *et al.*, 1994
	Increase	-	Yamamoto *et al.*, 1998
	-	Increase	Schroedl *et al.*, 2001
	Increase	Increase	Ganheim *et al.*, 2003
	-	Increase	Corrigan *et al.*, 2007
Pasteurella multocida	Increase	Increase	Dowling *et al.*, 2002
Histophilus somni	-	Increase	McNair *et al.*, 1997

-: not investigated

4. IMMUNOMODULATION BY DRUGS IN BOVINE VETERINARY MEDICINE

Immunopharmacology is a relatively recent research field that aims to manipulate the immune system by modifying the endogenous immune responses to the benefit of the host in the treatment of diseases (Hadden and Kishimoto, 1993). One of its subdisciplines studies the influence of pharmacological agents on the innate immune response. Particularly in human medicine, the first reports on such beneficial effects of drugs have initiated intensive research regarding immunomodulatory drugs. Additionally, the indication that certain antimicrobial agents can exert immunomodulatory properties has led to high prospects of this topic. Labro (2000) even described these antibiotics as "a therapeutic need for the third millennium" with stress on the use in immunocompromised patients. However, it should be remarked that immunomodulation differs from immunosuppression or anti-inflammation and that it does not strictly refer to beneficial effects (Hamilton-Miller, 2001; Kanoh and Rubin, 2010). Van Vlem *et al.* (1996) distinguished immuno-enhancing antibiotics from immuno-depressing antibiotics. During this classification, one of the major problems regarding immunopharmacology was observed: the inconsistency of results. One of the causes, contributing to these inconsistencies, is the diversity of methods used to assess immunomodulatory effects. In this respect, methods ranging from *in vitro* and *ex vivo* studies on the alteration of the phagocytic function of leukocytes to the *in vivo* evaluation of the influence on the release of pro-inflammatory cytokines have been applied.

In veterinary medicine, the choice of a pharmacological agent remains nowadays mainly based on its direct activity. Nevertheless, the immunomodulatory properties of drugs could provide an added value in the treatment of an animal. Mulcahy and Quinn (1986) reviewed the application of immunomodulators in veterinary medicine in the 1980s. Based on more recent data, an overview of the current knowledge on immunomodulatory drugs will be provided, with emphasis on bovine veterinary medicine. In this respect, bovine *in vitro*, *ex vivo* and *in vivo* studies using **antimicrobial drugs**, **non-steroidal anti-inflammatory drugs** (NSAIDs) and **corticosteroids** were taken into account.

4.1. BOVINE *IN VITRO* STUDIES WITH RESPECT TO IMMUNOMODULATORY DRUGS

In vitro studies generally focus on the following endpoints to study immunomodulatory properties of drugs: phagocytosis, respiratory burst, transcription and/or release of inflammatory mediators and neutrophil recruitment and apoptosis. These parts of the innate immune response are highly important in the defence against invading micro-organisms, and the subsequent initiation of the acute-phase response.

The main phagocytic cells include neutrophils and monocytes/macrophages. Due to their rapid and abundant recruitment, neutrophils act as the first line of defence in case of an inflammatory event. During such an insult, local resident macrophages produce multiple factors of inflammation, including cytokines and chemokines. These products stimulate the adhesion of phagocytes to endothelial cells. The adhesion is initiated by a weak selectin-mediated interaction between both cell types, resulting in rolling of neutrophils. Subsequent presentation of chemokines on the endothelial cell surface will induce neutrophil integrin activation. The consequence of this inside-out signaling, is a firm phagocyte adhesion to the endothelium (Kinashi, 2005). Diapedesis is the next step in the neutrophil recruitment, and is mainly characterized by paracellular migration of phagocytes through the vascular endothelium (Muller, 2003). This is followed by the migration towards the inflammatory site under the influence of a chemotactic gradient, which consists of microbial components and endogenous chemoattractants, including complement factor 5a, leukotriene B_4 (LTB_4), platelet activating factor and neutrophil-active chemokines, such as IL-8. Due to the concurrent exposure to priming agents, neutrophil functions are potentiated, which include the manifestation of powerful antimicrobial activities. Monocytes are concomitantly recruited by chemotaxis, yet not as fast and extensively as neutrophils (Silva, 2010).

Both types of leukocytes then continue their phagocytic activities by the recognition of their targets through multiple ligand-receptor interactions. Various opsonins, including immunoglobulins, collectins and complement components are involved in opsonin-dependent phagocytosis, while direct target recognition (opsonin-independent phagocytosis) is another possible trigger of engulfment (van Lookeren *et al.*, 2007). The internalized particle is subsequently delivered to the phagosome, which matures into a

phagolysosome through vesicle-mediated delivery (degranulation process) of various antimicrobial effectors, including proteases, antimicrobial peptides and lysozyme (Garin *et al.*, 2001). This phagolysosome is the ultimate location of killing of pathogens and in this respect, two different, yet synergistically acting mechanisms can be distinguished. The first mechanism – the oxygen-independent – covers a broad array of antimicrobial proteins and peptides including bactericidal/permeability-increasing protein, lactoferrin, lysozyme, and defensins. The oxygen-dependent mechanism – the respiratory burst – on the other hand, refers to the assemblage of the NADPH-oxidase complex, resulting in its activation. Accordingly, the phagocytes' oxygen consumption increases with simultaneous generation of superoxide anion radicals ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2). In the presence of myeloperoxidase, which is released during the degranulation process, hypochloric acid (HOCl) and chloramines are generated. Another antimicrobial system of phagocytic cells is the inducible nitric oxide synthase (iNOS) pathway, which generates nitric oxide (NO) radicals. Both the NADPH-oxidase and the iNOS pathway occur in both types of phagocytes, although the first pathway is more pronounced in neutrophils, and the latter in macrophages (Nathan and Shiloh, 2000). The final step of the phagocytosis process is the digestion of the debris by hydrolases and other lytic enzymes present in the phagolysosome.

Phagocytosis-induced reactive oxygen species (ROS) are highly antimicrobial, but can also damage the surrounding tissue, resulting in an amplification of the inflammatory reaction. Additionally, the release of proteolytic compounds from necrotic neutrophils can contribute to self-perpetuating inflammatory injury (Buret, 2010). During neutrophil apoptosis, on the other hand, the integrity of the membrane and the organelles remains preserved. Overall, the following immunomodulatory effects of drugs are regarded to be beneficial: stimulation of phagocytosis, inhibition of the respiratory burst and ROS, downregulation of pro-inflammatory cytokines, prevention of excessive neutrophil infiltration (reduced chemotaxis, LTB_4 and IL-8) and induction of neutrophil apoptosis. With respect to the determination of these properties for different drugs, several *in vitro* studies using bovine cells from healthy animals have been performed. The underlying mechanisms, on the other hand, remain largely unknown. Table 3 provides an overview of the findings regarding antimicrobial drugs, NSAIDs, corticosteroids and miscellaneous drugs.

As can be concluded from Table 3, particularly **antimicrobial drugs** were studied *in vitro* (63% of the reported drugs: 36 antibiotics, belonging to 9 classes). Indeed, therapeutic compounds that generate both anti-bacterial as well as immunomodulatory effects are of great interest for the treatment of bacteria-induced inflammatory diseases (Buret, 2010). Conversely, several antibiotics have been reported to reduce phagocytic and bactericidal activities of neutrophils, in this way impairing the host's defence mechanism. Knowledge on the effect of an antibiotic on the innate immune response can consequently serve as a secondary aid in the selection of an antibiotic for antimicrobial therapy (Paape *et al.*, 1991). In this respect, it should also be kept in mind that antimicrobial drugs that are effective against the bacterial cell wall (like penicillins and cephalosporins) result in a greater release of LPS than those that affect microbial protein synthesis (such as macrolides) (Bentley *et al.*, 2002).

Besides antibiotics, several **anti-inflammatory drugs** were evaluated with respect to their immunomodulatory properties (NSAIDs and corticosteroids: 14 and 12% of the drugs included in Table 3, respectively). The reported effects occur independently of their direct anti-inflammatory actions, which encompass the inhibition of COX by NSAIDs and of PLA_2 by glucocorticoids, with subsequent blocking of prostaglandins, thromboxanes and/or leukotrienes (Fig. 6). Their immunomodulatory effects, on the other hand, include the inhibition of NF-κB-related gene transcription (Brattsand and Linden, 1996; Tegeder *et al.*, 2001). This was indeed confirmed *in vitro* for acetylsalicylic acid, flunixin meglumine and dexamethasone (Table 3) (Kiku *et al.*, 2002; Malazdrewich *et al.*, 2004a; Myers *et al.*, 2010).

Table 3. Evaluation of *in vitro* immunomodulatory effects of antimicrobial drugs, NSAIDs, corticosteroids and miscellaneous drugs using bovine cells.
Blank cells represent non-investigated effects.

Drug	Cell type	EFFECTS					Reference
		Phagocytosis	Respiratory burst (RB) and reactive oxygen species (ROS)	Pro-inflammatory cytokines	Chemotaxis, LTB_4 and IL-8	Apoptosis	
ANTIMICROBIAL DRUGS							
Aminoglycosides							
Amikacin	Milk neutrophil	Decrease					Paape *et al.*, 1991
Dihydrostreptomycin	Blood neutrophil		NE				Hoeben *et al.*, 1998a
Gentamycin	Milk neutrophil	Decrease					Paape *et al.*, 1991
Neomycin	Blood neutrophil		NE				Hoeben *et al.*, 1998a
Spectinomycin	Milk neutrophil	NE					Paape *et al.*, 1991
Streptomycin	Milk neutrophil	NE					Paape *et al.*, 1991
Tobramycin	Milk neutrophil	Decrease					Paape *et al.*, 1991
B-lactam antibiotics							
Ampicillin	Milk neutrophil	NE					Paape *et al.*, 1991
	Blood neutrophil		Increase ROS scavenging				Hoeben *et al.*, 1998a
Benzylpenicillin	Milk neutrophil		Decrease RB				Hoeben *et al.*, 1997a
	Blood neutrophil		Decrease RB – Increase ROS scavenging				Hoeben *et al.*, 1998a
Carfecillin	Milk neutrophil	Decrease					Paape *et al.*, 1991
Ceftiofur	Milk neutrophil		Increase RB				Hoeben *et al.*, 1997a
	Blood neutrophil		Increase RB – Increase ROS scavenging				Hoeben *et al.*, 1998a
						NE	Fischer *et al.*, 2011
Cephacetrile	Milk neutrophil	Decrease					Paape *et al.*, 1991
Cephapirin	Milk neutrophil	Decrease					Paape *et al.*, 1991
	Blood neutrophil	NE	Decrease RB[a] – Increase ROS scavenging[a]				Dosogne *et al.*, 1998
Cloxacillin	Milk neutrophil	NE					Paape *et al.*, 1991
	Blood neutrophil		NE				Hoeben *et al.*, 1998a
Mecillinam	Blood neutrophil	NE	Decrease RB[a] – Increase ROS scavenging[a]				Dosogne *et al.*, 1998

Drug	Cell type	Phagocytosis	RB and ROS	Cytokines	Chemotaxis	Apoptosis	Reference
Nafcillin	Milk neutrophil	NE					Paape *et al.*, 1991
Penicillin	Milk neutrophil	NE					Paape *et al.*, 1991
	Blood neutrophil					NE	Fischer *et al.*, 2011
Chloramphenicol and analogs							
Chloramphenicol	Milk neutrophil	Decrease					Paape *et al.*, 1990
			Decrease RB				Hoeben *et al.*, 1997a
	Blood neutrophil	Decrease	Decrease RB				Hoeben *et al.*, 1997b
Florfenicol	Milk neutrophil	NE					Paape *et al.*, 1990
Thiamphenicol	Milk neutrophil	NE					Paape *et al.*, 1990
Fluoroquinolones							
Ciprofloxacin	Milk neutrophil	NE					Paape *et al.*, 1991
Danofloxacin	Milk neutrophil		Decrease RB				Hoeben *et al.*, 1997a
	Blood neutrophil		Increase ROS scavenging				Hoeben *et al.*, 1997b
Enrofloxacin	Milk neutrophil		Increase RB				Hoeben *et al.*, 1997a
	Blood neutrophil		Increase RB				Hoeben *et al.*, 1997b
Flumequine	Milk neutrophil	NE					Paape *et al.*, 1991
Lincosamides							
Lincomycin	Blood neutrophil		NE				Hoeben *et al.*, 1998a
Macrolides							
Erythromycin	Milk neutrophil	NE					Paape *et al.*, 1991
			NE				Hoeben *et al.*, 1997a
	Blood neutrophil	Decrease	NE				Hoeben *et al.*, 1997b
Oleandomycin	Blood neutrophil		NE				Hoeben *et al.*, 1998a
Spiramycin	Milk neutrophil	NE					Paape *et al.*, 1991
			NE				Hoeben *et al.*, 1997a
	Blood neutrophil	Decrease	NE				Hoeben *et al.*, 1997b
Tilmicosin	Alveolar macrophage	NE			NE on chemotaxis		Brumbaugh *et al.*, 2002
	Blood neutrophil					Induction	Chin et al., 2000
					Decrease LTB_4 synthesis	Induction	Lee *et al.*, 2004
	Macrophage	Increase efferocytosis					Chin *et al.*, 2000
						NE	Lee *et al.*, 2004
Tulathromycin	Blood neutrophil			Decrease NF-κB activation	Decrease IL-8 mRNA	Induction	Fischer *et al.*, 2011
					Decrease LTB_4 secretion		Fischer *et al.*, 2014
	Macrophage					NE	Fischer *et al.*, 2011
		Increase efferocytosis			Decrease IL-8 secretion	Induction	Fischer *et al.*, 2013

Drug	Cell type	Phagocytosis	RB and ROS	Cytokines	Chemotaxis	Apoptosis	Reference
Tylosin	Milk neutrophil	NE					Paape *et al.*, 1991
	BME-UV cell line			Downregulation IL-1 Downregulation IL-6 NE on TNF-α	NE on IL-8		Mazzilli and Zecconi, 2010
Polymyxins							
Polymyxin B	Alveolar macrophage			Decrease TNF-α Decrease IL-1β Decrease TNF-α mRNA Decrease IL-1β mRNA			Yoo *et al.*, 1995
Sulphonamides							
Sulphadiazine	Milk neutrophil		NE				Hoeben *et al.*, 1997a
	Blood neutrophil		NE				Hoeben *et al.*, 1997b
Tetracyclines							
Doxycycline	Milk neutrophil	Decrease					Paape *et al.*, 1991
	Blood neutrophil		Decrease RB – Increase ROS scavenging				Hoeben *et al.*, 1998a
Minocycline	Milk neutrophil	Decrease					Paape *et al.*, 1991
Oxytetracycline	Milk neutrophil	NE					Paape *et al.*, 1991
			NE				Hoeben *et al.*, 1997a
	Blood neutrophil		Increase RB[a]				Myers *et al.*, 1995
		Decrease	NE				Hoeben *et al.*, 1997b
						NE	Fischer *et al.*, 2011
NSAIDs							
Acetominophen	Milk neutrophil	Decrease					Paape *et al.*, 1991
Acetylsalicylic acid	Milk neutrophil	Increase					Paape *et al.*, 1991
	Whole blood			Decrease TNF-α mRNA			Myers *et al.*, 2010
Centrophenoxine	Milk neutrophil	Increase					Paape *et al.*, 1991
Flunixin meglumine	Whole blood			Decrease TNF-α mRNA			Myers *et al.*, 2010
Ibuprofen	Milk neutrophil	Decrease					Paape *et al.*, 1991
Indomethacin	Milk neutrophil	Decrease					Paape *et al.*, 1991
	Milk macrophage				NE on chemotaxis		Politis *et al.*, 1991
	Whole blood			NE on TNF-α mRNA			Myers *et al.*, 2010
Phenylbutazone	Milk neutrophil	Decrease					Paape *et al.*, 1991
PTPBS[b]	Whole blood			Decrease TNF-α mRNA			Myers *et al.*, 2010
CORTICOSTEROIDS							
6α-methylprednisolone	Blood neutrophil		NE				Hoeben *et al.*, 1998b
Betamethasone	Blood neutrophil		NE				Hoeben *et al.*, 1998b
Cortisone	Blood neutrophil		NE				Hoeben *et al.*, 1998b

Drug	Cell type	Phagocytosis	RB and ROS	Cytokines	Chemotaxis	Apoptosis	Reference
Dexamethasone	Blood neutrophil	Decrease					Ohman *et al.*, 1987
			NE				Hoeben *et al.*, 1998b
						NE	Chin *et al.*, 2000
	PBMC[c]			Decrease TNF-α mRNA Decrease IL-1β mRNA Decrease IL-6 mRNA			Kiku *et al.*, 2002
	Blood neutrophil					Decrease	Chang et al., 2004
	Alveolar macrophage			Decrease TNF-α Decrease IL-1β Decrease TNF-α mRNA Decrease IL-1β mRNA	Decrease IL-8 Decrease IL-8 mRNA		Malazdrewich *et al.*, 2004a
	Whole blood			NE on TNF-α mRNA			Myers *et al.*, 2010
Hydrocortisone	Blood neutrophil		NE				Hoeben *et al.*, 1998b
Prednisolone	Blood neutrophil		NE				Hoeben *et al.*, 1998b
OTHERS							
Chlorpromazine	PBMC[c]			Decrease TNF-α mRNA Decrease IL-1β mRNA NE on IL-6 mRNA			Kiku *et al.*, 2002
Pentoxifylline	PBMC[c]			NE on TNF-α mRNA NE on IL-1β mRNA NE on IL-6 mRNA			Kiku *et al.*, 2002
	Alveolar macrophage			Decrease TNF-α Decrease IL-1β Decrease TNF-α mRNA NE on IL-1β mRNA	Decrease IL-8 NE on IL-8 mRNA		Malazdrewich *et al.*, 2004a
Rolipram	Alveolar macrophage			Decrease TNF-α Decrease IL-1β NE on TNF-α mRNA NE on IL-1β mRNA	NE on IL-8 NE on IL-8 mRNA		Malazdrewich *et al.*, 2004a
SB203580[d]	Alveolar macrophage			Decrease TNF-α NE on IL-1β NE on TNF-α mRNA NE on IL-1β mRNA	Decrease IL-8 NE on IL-8 mRNA		Malazdrewich *et al.*, 2004a
Tetrahydropapaveroline	Alveolar macrophage			Decrease TNF-α Decrease IL-1β Decrease TNF-α mRNA Decrease IL-1β mRNA	Decrease IL-8 Decrease IL-8 mRNA		Malazdrewich *et al.*, 2004a
Thalidomide	Alveolar macrophage			NE on TNF-α NE on IL-1β NE on TNF-α mRNA NE on IL-1β mRNA	NE on IL-8 NE on IL-8 mRNA		Malazdrewich *et al.*, 2004a

NE: no effect; [a]: only at high concentrations; [b]: 4-[5-phenyl-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzene sulfonamide (a specific COX-2 inhibitor); [c]: peripheral blood mononuclear cells; [d]: a bicyclic imidazole

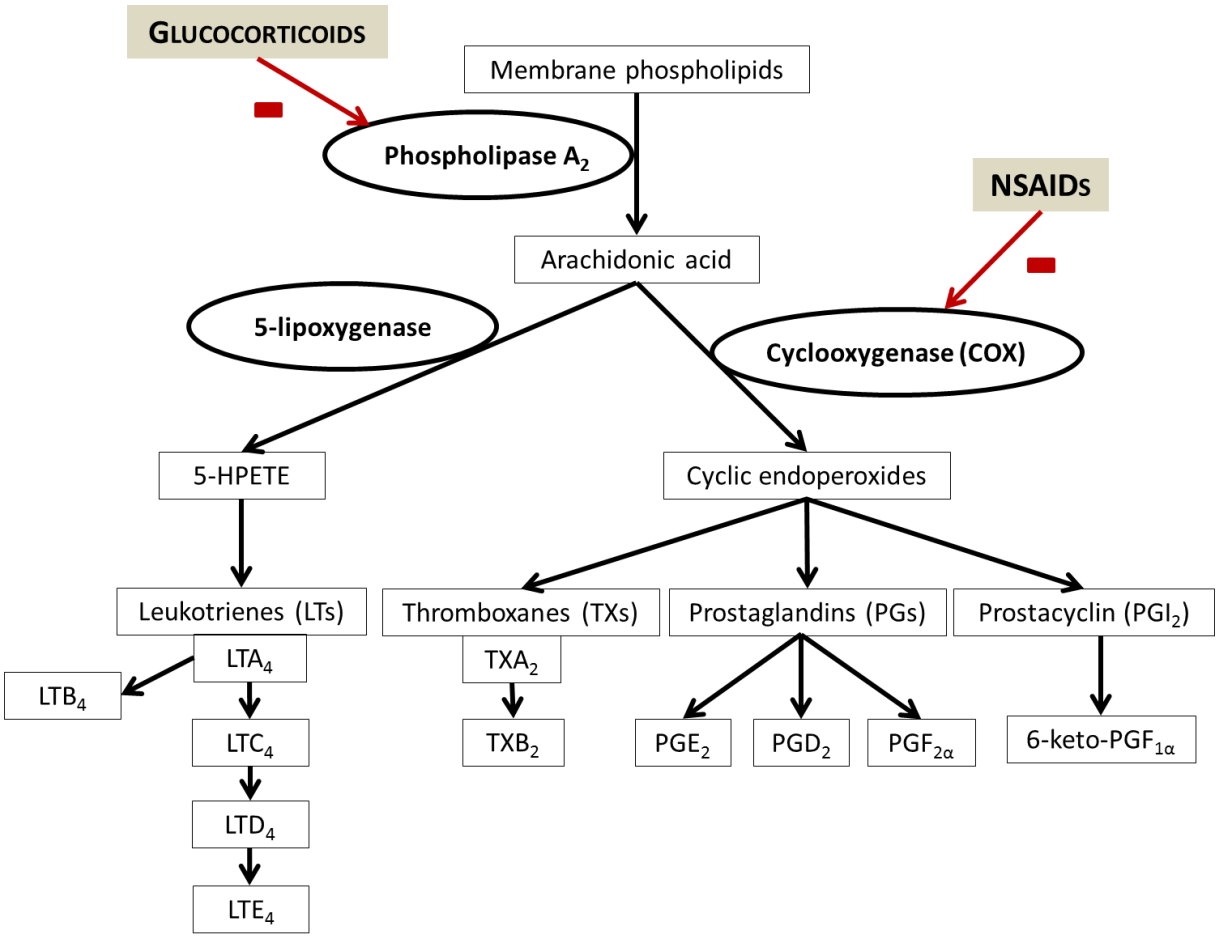

Figure 6. Direct anti-inflammatory actions of NSAIDs and corticosteroids: inhibition of the enzymatic production of eicosanoids (based on Adams, 2001)

In addition to antibiotics, NSAIDs and corticosteroids, a number of drugs belonging to **miscellaneous classes** were also studied with respect to their immunomodulatory properties (11% of the reported drugs in Table 3). The choice of these drugs was based on conclusions from earlier studies. Chlorpromazine, for example, is a psychotropic drug that has been demonstrated to inhibit TNF-α release in mice (Gadina *et al.*, 1991). Additionally, the hemorrheologic agent pentoxifylline, the antidepressant rolipram, the imidazole SB203580, the antioxidant tetrahydropapaveroline and the sedative thalidomide have been mentioned in this context (Sampaio *et al.*, 1991; Eugui *et al.*, 1994; Lundblad *et al.*, 1995; Badger *et al.*, 1996; Korhonen *et al.*, 2013). *In vitro* studies using bovine cells indeed established the inhibition of pro-inflammatory cytokines by the former drugs, with the exception of thalidomide (Kiku *et al.*, 2002; Malazdrewich *et al.*, 2004a). Their application in bovine veterinary medicine, on the other hand, is rather questionable and even unlikely.

Regarding the applied cell types, milk and blood derived neutrophils are clearly the most frequently studied leukocytes. Accordingly, neutrophils have been reported to be a key player in the pathogenesis of for instance bovine respiratory disease and mastitis, based on their role in the production of inflammatory mediators, recruitment of other leukocytes and tissue damage (Aulik *et al.*, 2010; Buret, 2010).

As can be concluded from Table 3, several drugs inhibit the *phagocytic properties* of bovine leukocytes. This refers to the formerly mentioned distinction between immuno-enhancing and immuno-depressing drugs by Van Vlem *et al.* (1996). Only the NSAIDs acetylsalicylic acid and centrophenoxine have been confirmed to increase phagocytosis in bovine neutrophils. In human medicine, on the other hand, several antimicrobial drugs such as erythromycin, chloramphenicol and nafcillin have been reported to stimulate the phagocytic function (Labro *et al.*, 2000). With respect to efferocytosis – the clearance of apoptotic neutrophils by macrophages – both tilmicosin and tulathromycin have been demonstrated to exert an enhancing effect (Chin *et al.*, 2000; Fischer *et al.*, 2013). As mentioned before, such inconsistencies in *in vitro* results are highly frequent in immunopharmacology. Many controversies arise from the use of non-standardised techniques, cells obtained from various origins or various cell subsets (Labro, 1993). This is also clear from Table 3, as cephapirin, for example, decreased phagocytosis in milk neutrophils, while it had no effect in blood derived neutrophils. Additionally, Fischer *et al.* (2011) reported no influence of tulathromycin on apoptosis of macrophages, whereas in a more recent study (2013), these authors did observe the induction of apoptosis in macrophages by tulathromycin. The experimental protocols used to assess the immunomodulatory properties of the drugs listed in Table 3 were indeed rather diverse. For instance, most studies applied higher concentrations than achievable in the animal, while others evaluated therapeutic doses (Paape *et al.*, 1991; Hoeben *et al.*, 1997a, 1997b, 1998; Fajt *et al.*, 2000). Nevertheless, authors using high drug concentrations claim that after local and repeated treatment much higher concentrations can be achieved in certain body compartments such as the udder. As a result, the influence of concentrations even up to 1000 µg/mL (unachievable *in vivo*) were frequently tested (Hoeben *et al.*, 1997b). It should be mentioned, however, that high drug concentrations can result in a reduced viability of cells as well, as reported for oxytetracycline by Hoeben *et al.* (1997b).

Regarding the influence of antimicrobial drugs on the *transcription and/or release of pro-inflammatory cytokines*, only data for one macrolide (tylosin) and polymyxin B are available from bovine *in vitro* studies (Yoo *et al.*, 1995; Mazzilli and Zecconi, 2010). The choice of the former class can be related to the results from *in vitro* experiments using innate immune cells from humans and laboratory animals (Khan *et al.*, 1999; Araujo *et al.*, 2002; Cao *et al.*, 2006). More specifically, these studies demonstrated an inhibition of TNF-α, IL-1β and IL-6 by azithromycin, clarithromycin, telithromycin, tilmicosin and/or tylosin. In this context, the involvement of NF-κB inactivation by macrolides has been suggested (Giamarellos-Bourboulis, 2008). Besides the impact on cytokines, an influence on neutrophil function, migration, infiltration and accumulation as well as on mucus (hyper)secretion and bacterial biofilm formation and quorum sensing has been reported for macrolides in humans and other animal species (Čulić *et al.*, 2001; Parnham, 2005; Ou et al., 2008). The effect of polymyxin B was evaluated since this antimicrobial drug interacts with the lipid A component of LPS, ultimately resulting in a reduced production of pro-inflammatory cytokines (Coyne and Fenwick, 1993). This hypothesis was indeed confirmed by Yoo *et al.* (1995) using bovine alveolar macrophages. The systemic administration of polymyxin B in the animal, on the other hand, is rather controversial, due to its nephrotoxic and neurotoxic side effects at antibacterial doses (Kelmer, 2009). Nevertheless, *in vivo* studies in goats and horses demonstrated that lower doses can effectively reduce clinical signs of experimentally induced endotoxemia, as well as TNF-α and/or IL-6 concentrations (van Miert et al., 1997; Morresey and Mackay, 2006). In this respect, a cautious approach for the treatment of animals in the clinical setting is highly recommended.

The corticosteroid dexamethasone was studied by different research groups. From the results of these *in vitro* studies, it can be concluded that dexamethasone is a positive control immunomodulatory drug with respect to the transcription and release of pro-inflammatory cytokines (Kiku *et al.*, 2002; Malazdrewich *et al.*, 2004a). Myers *et al.* (2010), on the other hand, did not detect any effect of dexamethasone on TNF-α mRNA expression. This unexpected observation could be attributed to the use of a rather low dose of the corticosteroid for whole blood cultures, in contrast to doses applied in experiments with purified innate immune cells.

4.2. BOVINE *EX VIVO* STUDIES WITH RESPECT TO IMMUNOMODULATORY DRUGS

Ex vivo studies with respect to immunomodulatory drugs refer to the *in vivo* treatment of healthy animals, followed by the collection of selected cell types and subsequent *in vitro* experiments. As a consequence, the ability of therapeutic drug doses to modulate innate immune functions is studied, instead of empirically selected – often supratherapeutic – doses. Table 4 gives an overview of such *ex vivo* bovine experiments, applied to evaluate the immunomodulatory properties of antibiotics, NSAIDs and corticosteroids. It is clear that *ex vivo* studies have been performed notably less frequent in comparison with *in vitro* studies. Only Donalisio *et al.* (2013) studied the effect of flunixin meglumine and ketoprofen on TNF-α production, which was inhibited by these NSAIDs. Nevertheless, in addition to the *ex vivo* studies in healthy animals, other authors based their research on experimentally infected animals that received an *in vivo* treatment post infection (Goubau and Buret, 2000; Fajt *et al.*, 2003). Such studies were not included in Table 4, as distinguishing between a bacterial effect and an influence of the drug on the immune function is not possible in these settings. From this point of view, the experimental protocol applied by Bednarek *et al.* (1999) might provide more advantages. More specifically, these authors induced local lung inflammation in calves by the injection of sterile mineral oil into the diaphragmatic lobus of the right lung. The calves then received a treatment with either the NSAID meloxicam or the corticosteroid flumethasone, after which blood samples were collected to evaluate the *ex vivo* ability of innate immune cells to produce TNF-α. The results of this study showed that both meloxicam and flumethasone significantly inhibited this cytokine's release. Additionally, both drugs had no effect on the phagocytic activity of blood phagocytes (Bednarek *et al.*, 1999).

Table 4. Evaluation of *ex vivo* immunomodulatory effects of antimicrobial drugs, NSAIDs and corticosteroids using samples derived from healthy bovines.

	DRUG					EFFECTS					
Drug	**Dose (mg/kg BW)**	**TOA (h)**	**ROA**	**TBC (h)**	**Cell type**	**Phagocytosis**	**Respira-tory burst**	**Pro-inflammatory cytokines**	**Chemotaxis, LTB_4 and IL-8**	**Apoptosis**	**Reference**
Antimicrobial drugs											
Fluoroquinolones											
Danofloxacin	6	0	SC	12; 26; 60	Blood neutrophil	NE	NE		NE on chemotaxis	-	Fajt *et al.*, 2000
Marbofloxacin	2	0; 24; 48; 72; 96	SC	120	Whole blood	-	Decrease	-	-	-	Spehner *et al.*, 1996
Macrolides											
Tilmicosin	10	0	SC	12; 26; 60	Blood neutrophil	NE	NE	-	NE on chemotaxis	-	Fajt *et al.*, 2000
NSAIDs											
Flunixin meglumine	2.2	0; 12; 36; 60	IV	1; 13; 37; 61; 85	Whole blood	-	-	Decrease TNF-α	NE on LTB4 Decrease IL-8	-	Donalisio *et al.*, 2013
Ketoprofen	3	0; 12; 36; 60	IV	1; 13; 37; 61; 85	Whole blood	-	-	Decrease TNF-α	NE on LTB4 Decrease IL-8	-	Donalisio *et al.*, 2013
Corticosteroids											
Dexamethasone	SA[a]: 0.08	0	IM	13; 37; 61;	Blood neutrophil	-	-	-	Increase chemotaxis	-	Anderson *et al.*, 1999
	LA[b]: 0.25	37	IM	109; 181; 253							
	0.1	G1[c]: 0 G2[c]: 0 G3[c]: 0; 6 G4[c]: 0; 6; 12	IM	G1: 3 G2: 6 G3: 12 G4: 24	Blood neutrophil	-	-	-	-	Decrease	Chang *et al.*, 2004

TOA: time(s) of administration; ROA: route of administration; TBC: time of blood collection relative to the first drug administration; SC: subcutaneous; IV: intravenous; IM: intramuscular; NE: no effect; -: not investigated; [a]: short acting; [b]: long acting; [c]: group

4.3. BOVINE *IN VIVO* STUDIES ON IMMUNOMODULATORY DRUGS USING LPS INFLAMMATION MODELS

As *in vitro* and *ex vivo* studies with experimentally fixed systems do not truly reflect the dynamics and multiple interactions that occur *in vivo*, inflammation models in the absence of active bacteria can provide more information on the immunomodulatory properties of drugs (Labro, 1993; Bednarek *et al.*, 2003; Buret, 2010). In this respect, LPS-induced inflammation models can be put forward and could be advisable. An overview of such bovine *in vivo* studies concerning the effects of drugs on body temperature, pulmonary response, clinical score and levels of cytokines and acute-phase proteins following an LPS challenge is provided in Table 5. Similarly to Table 4, the results of studies with natural or experimentally induced infections are not included in Table 5, since the presence of actively invading and growing microorganisms complicates the interpretation of the effect of immunomodulatory drugs. In comparison with *in vitro* studies, it is clear that the number of drugs evaluated *in vivo* is again remarkably lower. Moreover, emphasis was rather placed on the clinical outcome. With respect to **antimicrobial drugs**, only the immunomodulatory effect of tilmicosin was studied in cattle by Reuter *et al.* (2008). It should be mentioned, however, that the main aim of these authors was to evaluate the effects of the dietary energy source and level on the immune function, and that the influence of tilmicosin was only assessed additionally. The decrease of SAA concentrations, for example, was merely observed in the group receiving a restricted amount of a 70% concentrate diet. Also in other animal species, data from *in vivo* studies on the modulation of pro-inflammatory cytokine release by antimicrobial drugs are rather scarce, and principally limited to mice and rats (Ianaro *et al.*, 2000; Tkalcevic *et al.*, 2006; Leiva *et al.*, 2007; Wyns *et al.*, 2015b).

Regarding **NSAIDs and corticosteroids**, mostly commonly used drugs were studied. Overall, the anti-inflammatory drugs decreased the clinical score following the LPS challenge. This clinical score generally encompassed the occurrence of depression and anorexia, as these are important signs in the early detection of illness. Regarding the influence on body temperature and pulmonary response, the evaluated drugs had nearly no effect. With respect to the studies of Semrad (1993a, 1993b), this might be related to the administration of the drug 15 min after the IV LPS bolus, since an inflammatory reaction is already initiated at this time. The administration of dexamethasone prior to the challenge indeed resulted in

a more pronounced effect, including a reduction of the pulmonary response (Olson and Brown, 1986; Ohtsuka *et al.*, 1997a). It can therefore be concluded that the time of drug administration is of major importance with respect to the effect on the clinical outcome. Pre-treatment of calves with dexamethasone did not affect the body temperature, which might be attributed to the rather slow action of corticosteroids, due to the need for lipocortin synthesis (Hirata, 1983; Ohtsuka *et al.*, 1997a). Nevertheless, the results of the study of Ohtsuka *et al.* (1997a) concerning TNF-α inhibition confirm our previous conclusion from bovine *in vitro* studies regarding dexamethasone being a positive control immunomodulatory drug.

Similarly to the *in vitro* studies, a number of drugs belonging to **miscellaneous classes** were studied *in vivo* with respect to their immunomodulatory properties. Besides chlorpromazine and pentoxifylline, also dimethyl sulfoxide (DMSO) and two lazaroids (U74389F and tirilazad mesylate) were evaluated. The anti-inflammatory compound DMSO was selected by Semrad (1993b) based on its antithrombogenic, endothelial sparing and free radical scavenging capabilities. The lazaroids, on the other hand, are antioxidants that combine certain properties of NSAIDs and corticosteroids, without the side effects of the latter. Moreover, tirilazad mesylate was evaluated by Rose and Semrad (1992) as this drug for the treatment of central nervous system trauma would have the capacity to mitigate the adverse reactions induced by the entrance of endotoxin into the circulation. Surprisingly, from these five drugs, chlorpromazine had the most pronounced influence on the LPS-induced inflammatory reaction (Ohtsuka *et al.*, 1997a). More specifically, the body temperature, pulmonary response, clinical score and TNF-α levels were all significantly decreased by this psychotropic drug. Nevertheless, its side effects, including tachycardia, hypothermia and sedation, cannot be neglected. It should also be mentioned that Semrad (1993b) did not report the effects of all drugs on the studied parameters, which complicates the interpretation of this experiment (i.e. NR or not reported in Table 5).

Table 5. Bovine *in vivo* studies using LPS-induced inflammation to evaluate the immunomodulatory properties of antimicrobial drugs, NSAIDs, corticosteroids and miscellaneous drugs.

Drug	DRUG				EFFECTS						Reference
	Bolus (mg/kg BW)	Infusion (duration)	ROA	TOA (h)	Body temperature	Pulmonary response	Clinical score	TNF-α	IL-6	SAA	
Antimicrobial drugs											
Macrolides											
Tilmicosin	10		SC	-48	NE	-	-	Trend to increase	NE	Decrease[a]	Reuter *et al.*, 2008
NSAIDs											
Flunixin meglumine	1.1		IV	+0.25	NE	NE	Decrease	-	-	-	Semrad, 1993a
Flunixin meglumine	1.1		IV	+0.25	NR	NR	Decrease	-	-	-	Semrad, 1993b
Ketoprofen	2.2		IV	+0.25	NE	NE	Decrease	-	-	-	Semrad, 1993a
Ketorolac	1.1		IV	+0.25	NE	NE	Decrease	-	-	-	Semrad, 1993a
Corticosteroids											
Dexamethasone	5	5 mg/kg BW (5 h: during endotoxemia)	IV	Bolus: -18; -1	-	Decrease	-	-	-	-	Olson and Brown, 1986
	0.3		IV	-1	NE	Decrease	Decrease	Decrease	-	-	Ohtsuka *et al.*, 1997a
Prednisolone	1.1		IV	+0.25	NR	NR	Decrease	-	-	-	Semrad, 1993b
Others											
Chlorpromazine	4		IV	-1	Decrease	Decrease	Decrease	Decrease	-	-	Ohtsuka *et al.*, 1997a
DMSO		0.5 g/kg BW (1.25 h)	IV	+0.25	NE	NR	Increase	-	-	-	Semrad, 1993b
Pentoxifylline	20		IV	-1	NE	NE	NE	NE	-	-	Ohtsuka *et al.*, 1997a
Tirilazad mesylate		1.5 mg/kg BW (0.08 h)	IV	G1[c]: -1 G2[c]: +1	NE	NE	Decrease	Decrease	-	-	Rose and Semrad, 1992 & Semrad *et al.*, 1993
U74389F[b]	1.5		IV	+0.25	NR	NE	NE	-	-	-	Semrad, 1993b

ROA: route of administration; TOA: time of administration relative to LPS challenge; SC: subcutaneous; IV: intravenous; NE: no effect; NR: not reported; -: not investigated; [a]: in case of a specific diet; [b]: a lazaroid; [c]: group

5. RELEVANCE OF LIPOPOLYSACCHARIDE INFLAMMATION MODELS AND THE SELECTED DRUGS FOR BOVINE VETERINARY MEDICINE

The IV administration of LPS results in a series of clinical signs, which are rather non-specific: depression, anorexia and fever. As these signs are part of several bovine diseases, including pneumonia, septicemia and neonatal diarrhea, the possible contributing role of LPS in the development of these clinical entities can be put forward. The former sections are consequently not limited to fundamental research, as the use of LPS inflammation models with respect to the study of immunomodulatory drugs can provide valuable information for cattle practice. More specifically, such information can contribute to improved treatment strategies for LPS-associated diseases, resulting in economic, public and animal benefits.

Since immunopharmacology aims to manipulate the immune system to the benefit of the host, immunomodulatory drugs would ideally induce a more rapid and improved clinical recovery, resulting in enhanced animal welfare and better production results (Hadden and Kishimoto, 1993). In this context, drugs that exert both anti-bacterial as well as anti-inflammatory effects have been suggested to be most effective in treating bacteria-induced inflammatory diseases (Buret, 2010). Nevertheless, antimicrobial resistance is currently one of the leading health concerns in human and veterinary medicine (Pardon *et al.*, 2012a). Rational use of this class of drugs is therefore highly recommended. In this respect, the concomitant use of an antibiotic and an anti-inflammatory drug can provide synergistic or additive benefits. The choice between a corticosteroid and a NSAID, however, remains a controversial topic in bovine veterinary medicine (Lekeux and Van de Weerdt, 1997). More specifically, despite the side effects of corticosteroids, including suppression of the immune system and the adrenal gland, these drugs are still commonly preferred to NSAIDs by practitioners for their potential pharmacodynamic action.

In the present thesis, one drug of each class was selected to be evaluated with respect to its immunomodulatory properties. Firstly, **gamithromycin** was chosen since macrolides have been suggested to be the most promising antimicrobial drugs regarding immunomodulation. This 15-membered semi-synthetic azalide is registered in view of the therapeutic and preventive treatment of bovine respiratory disease, associated with *M.*

haemolytica, *P. multocida* and *H. somni*. The superior characteristics of this drug are its potency, as well as in its long half-life of elimination (51 h), which is reflected by the ease of use, as only a single SC administration is needed (Huang *et al.*, 2010).

Secondly, the corticosteroid **dexamethasone** was selected as a positive control immunomodulatory drug, based on the reported results from several bovine studies (Ohtsuka *et al.*, 1997a; Kiku *et al.*, 2002; Malazdrewich *et al.*, 2004a). As previously mentioned, veterinarians frequently decide on using this anti-inflammatory drug to elicit a fast clinical response in acute inflammatory diseases. Moreover, corticosteroids can be administered exclusively by veterinarians in Belgium.

Lastly, the NSAID **ketoprofen** was included for its previously reported beneficial effects in cattle (Semrad, 1993a; Donalisio *et al.*, 2013). Additionally, according to a study of Pardon *et al.* (2012a), ketoprofen is the most frequently used NSAID in Belgian white veal calves.

To the best of our knowledge, comparative *in vivo* studies regarding the immunomodulatory properties of antimicrobials drugs, corticosteroids and NSAIDs have not yet been performed in cattle. Moreover, LPS inflammation models to study these effects are currently rather limited and poorly standardized (Table 5).

SCIENTIFIC AIMS

The first reports suggesting beneficial effects of certain antimicrobial and (non)-steroidal anti-inflammatory drugs ((N)SAIDs) on the innate immune system have led to high prospects of the research field of immunomodulation. In this respect, the need for bovine inflammation models in the absence of active bacteria has been put forward repeatedly. Consequently, an LPS inflammation model in calves can be used to investigate the influence of drugs on the release of pro-inflammatory cytokines and acute-phase proteins, as well as on the induced clinical signs. Irrespective of clinical settings, it has been demonstrated that pre-treatment is necessary in *in vivo* experiments to elicit a maximum effect of the drug on the immune response.

Macrolides have been suggested to be the most promising antimicrobial drugs with respect to immunomodulation in humans and animals. The second generation macrolide gamithromycin (GAM) has been registered for the treatment of bovine respiratory disease, whereas data on its immunomodulatory properties are currently lacking. With respect to the use of anti-inflammatory drugs as ancillary therapy to bovine respiratory disease, the corticosteroid dexamethasone (DEX) and the NSAID ketoprofen (KETO) are frequently administered, irrespectively of the controversy regarding the choice between these drug classes. To date, it is insufficiently known to what extent DEX and KETO or their combination with GAM (GAM-DEX and GAM-KETO) can modulate the acute-phase response.

Therefore, the **GENERAL AIM** of this doctoral thesis was to evaluate the immunomodulatory properties of GAM, DEX and KETO alone, as well as their two-drug combinations in a standardized LPS inflammation model in calves. This information contributes to an improved, evidence-based treatment strategy, with respect to production results, animal welfare and responsible drug use.

In order to realize the general aim, the following **SPECIFIC AIMS** were postulated:

1) To develop and characterize an LPS inflammation model in calves with emphasis on inflammatory mediators and the appearance of different clinical signs *(Chapter 1)*.
2) To determine the pharmacokinetic properties of KETO following intramuscular administration in calves, in order to optimize the study design *(Chapter 2)*.
3) To assess the pharmacodynamic effects of GAM, DEX and KETO in the previously developed inflammation model *(Chapter 3)*.

SCIENTIFIC STUDIES

CHAPTER 1

Development of an intravenous lipopolysaccharide inflammation model in calves

Adapted from

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Abstract

Our objective was to develop an LPS inflammation model in calves to evaluate the acute-phase response with respect to the release of pro-inflammatory cytokines and acute-phase proteins, fever development and sickness behaviour. Fourteen 4-week-old male Holstein Friesian calves were included and randomly assigned to a negative control group (n = 3) and an LPS challenged group (n = 11). The latter received an intravenous bolus injection of 0.5 µg of LPS/kg body weight. Blood collection and clinical scoring were performed at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 18, 24, 28, 32, 48, 54 and 72 h post LPC challenge (p.c.). In the LPS group, the following clinical signs were observed successively: tachypnea (on average 18 min p.c.), decubitus (29 min p.c.), general depression (1.75 h p.c.), fever (5 h p.c.) and tachycardia (5 h p.c.). Subsequent to the recovery from respiratory distress, general depression was prominent, which deteriorated when fever increased. One animal did not survive LPS administration, whereas the other animals recovered on average within 6.1 h p.c. Moreover, the challenge significantly increased plasma concentrations of TNF-α, IL-6, SAA and Hp, with peaking levels at 1 h, 3.5 h, 24 h and 18 h p.c., respectively. The present LPS model was practical and reproducible, caused obvious clinical signs related to endotoxemia and a marked change in the studied inflammatory mediators, making it a suitable model to study the immunomodulatory properties of drugs in future research.

1. Introduction

As a part of the outer membrane of Gram-negative bacteria, LPS or endotoxin is involved in many infectious bovine diseases, including neonatal diarrhea and respiratory disease (Radostitis *et al.*, 2007; Pardon *et al.*, 2012b). Intravascular or peripheral exposure of host cells to LPS is a possible trigger of a complex series of non-specific, predetermined and well-orchestrated reactions, intending to control the Gram-negative bacterial infection (Conner *et al.*, 1989; Baumann and Gauldie, 1994): the acute-phase response. Nevertheless, the response can be excessive and subsequently result in detrimental effects to the host, like sepsis and septic shock (Peri *et al.*, 2010).

As a consequence of the marked similarity between the systemic effects of Gram-negative bacterial infections and those provoked by an exogenous LPS challenge, endotoxin models have been widely applied in veterinary research to study diverse aspects of the acute-phase response (Lillie, 1974; Redl *et al.*, 1993; Hodgson, 2006). More specifically, various experimental designs using different serotypes of LPS, ranging from high-dosed IV bolus injections to low-dose infusions over several hours, have been reported in calves (< 6 months) (Adams *et al.*, 1990; Kenison *et al.*, 1991; Gerros *et al.*, 1993; Semrad *et al.*, 1993; Kinsbergen *et al.*, 1994; Bieniek *et al.*, 1998; Kushibiki *et al.*, 2008). On the whole, data on the development of fever and the increased plasma concentrations of TNF-α are clear, emphasising the impact of contact with LPS. The duration and magnitude of the response, on the other hand, has been described to be related to the amount of circulating LPS (Gerros *et al.*, 1993). Remarkably, data on the release of IL-1β, IL-6 and acute-phase proteins are either lacking or less frequently reported in young calves following experimental endotoxemia. Also regarding sickness behaviour in these animals, limited information is available (Borderas *et al.*, 2008).

Therefore, our first objective was to develop an LPS inflammation model in 4-week-old calves, with inclusion of TNF-α, IL-1β, IL-6, SAA and Hp, which could be applied in further research regarding the immunomodulatory properties of (N)SAIDs and antimicrobials. Secondly, through applying this model, the authors attempted to design a clinical scoring system for the early detection of illness.

2. Materials and methods

2.1. Experimental animals

Fourteen healthy male Holstein Friesian calves were conveniently selected from local farms shortly after birth. The calves were conventionally reared on the farm, generally through separation from the mother and subsequent housing in individual pens. All animals received an oral treatment with paromomycin sulphate (100 mg/kg BW, sid, 10 days) (Gabbrovet® 70, Ceva Santé Animale, Brussels, Belgium) on the farm of origin, in order to reduce cryptosporidiosis related symptoms (Grinberg *et al.*, 2002). One week before the start of the experiment, at an average age of 22.6 ± 4.2 days, the calves were transferred to the Faculty of Veterinary Medicine. Upon arrival, the calves received a single treatment with 5 mg/kg BW enrofloxacin (Floxadil® 50 mg/ml, Emdoka, Hoogstraten, Belgium) s.c., to limit the spread of respiratory infections. During the acclimatization period, the animals were housed in individual pens on straw with ad libitum access to hay and fresh water. The calves were fed milk replacer three times a day, receiving a total of 5 L daily. After the morning feeding, 50 g of starter mix was given to the calves.

The animals' clinical condition was evaluated twice a day, including the determination of the rectal body temperature and visual inspection of the faeces. Analysis of faecal samples (Easy-Digest, Bio K 151, Bio-X, Jemelle, Belgium) revealed the presence of antigen of rotavirus, *E. coli* and *Cryptosporidium parvum* in a number of calves (36%, 21% and 29%, respectively). However, in order to avoid interference with the experiment, the calves were not treated pharmacologically during the week of acclimatization. In case of diarrhoea, the calves would receive electrolyte therapy. If illness would require an antimicrobial and/or an anti-inflammatory treatment, the calf would be excluded from the experiment.

At the end of the study, the calves were introduced in the teaching or experimental herd of our faculty.

2.2. Study protocol and sample collection

The day before the experiment, the calves were weighed (54.8 ± 7.0 kg), after which a 14 G indwelling catheter (Cavafix, B. Braun, Diegem, Belgium) was placed aseptically in the right jugular vein. A recovery period of at least 12 h was respected. After this period, the calves were randomly divided in two groups (average age 29.6 ± 4.2 days): a negative control group (CONTR; n = 3) and an LPS treated group (LPS; n = 11). The clinical condition at 0h was evaluated and a rectal body temperature ≥ 40 °C was handled as an exclusion criterion at this time.

Reference venous blood samples (0 h) for cytokines and acute-phase proteins were collected from the catheter and transferred into tubes containing EDTA. The calves in the LPS-group were subsequently challenged IV with 0.5 µg/kg BW ultrapure LPS (500 units/kg BW, *E. coli* serotype O111:B4, LPS-EB Ultrapure, InvivoGen, Toulouse, France) via the catheter, while the CONTR calves received a similar volume of saline. Blood samples for cytokines and acute-phase proteins analyses were collected at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 18, 24, 28, 32, 48, 54 and 72 h p.c. At all mentioned sampling points, rectal body temperature, respiratory rate and heart rate were recorded. The respiratory rate was assessed first, before entry of the pens. Subsequently, the rectal body temperature was measured using a digital thermometer, followed by heart rate determination. Animals were also clinically scored by a qualified veterinarian during the first 9 h of the experiment, including the evaluation of the presence of dyspnea, coughing, breathing sounds, mental state, position and appetite. If systemic shock symptoms would occur, the respective calf would be humanely euthanized, and subsequently necropsied. The investigators were not blinded to the treatment groups.

All blood samples were centrifuged at 1,000 × g for 15 min, after which the plasma was harvested and stored in aliquots at ≤ -70 °C for future analysis, with a maximum of 8 months.

All procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University) (EC 2009/153 and EC 2011/148).

2.3. Sample analyses for cytokines and acute-phase proteins

All plasma samples were analysed in duplicate for cytokines and acute-phase proteins using commercially available ELISAs. The assays for cytokines were validated prior to use, as suitability for plasma samples was not guaranteed by the manufacturer. The validation procedure consisted of the determination of imprecision parameters (intra- and inter-assay coefficients of variation (CV)) as well as method inaccuracies by evaluation of the linearity under dilution (Maddens *et al.*, 2010). Intra- and inter-assay CV values < 10% and < 15%, respectively, were assessed to be acceptable. For the evaluation of the inaccuracy, a recovery range of 80-120% for the linear dilutions was postulated. Additionally, the limit of quantification (LOQ) was calculated for these assays. To this end, the lowest cytokine concentration within the linear range of the standard curve was determined by evaluation of five standard curves for each cytokine.

Following the manufacturer's protocol for the Bovine TNF-α DuoSet (R&D Systems Europe, Abingdon, UK), an intra- and inter-assay CV of 5% and 8%, respectively, were obtained from the validation. The LOQ was set at 196 pg/mL. The validation procedure of the Bovine IL-1β and IL-6 Screening Sets (Thermo Fisher Scientific, Rockford, IL, USA), on the other hand, revealed the necessity of minor adjustments to the manufacturer's protocol. These adjustments implied a replacement of the suggested reagent diluent (4% bovine serum albumin in Dulbecco's phosphate buffered saline) by 5% Tween 20 in Dulbecco's phosphate buffered saline, an additional wash procedure after coating (three washes), an additional wash step after blocking (one wash), and an increased number of washes for the subsequent three wash steps (from three to five washes). By applying these modifications, the intra- and inter-assay CV of the IL-1β Screening Set were 8% and 12%, respectively, whereas for the IL-6 Screening Set these values were 7% and 11%, respectively. An LOQ of 125 and 286 pg/mL was calculated for IL-1β and IL-6, respectively.

The SAA (Phase SAA Assay, Tridelta Development Ltd., Maynooth, Ireland) and Hp (Haptoglobin Bovine ELISA, Alpco, Salem, NH, USA) assays were performed according to the manufacturer's protocol. The intra- and inter-assay CVs reported for the SAA ELISA were 7.5% and 12%, respectively, whereas for the Hp assay, both CVs were described to be < 10%.

The analytical sensitivities, calculated by the manufacturers, were 1.5 µg/mL and 3.64 ng/mL for the SAA and Hp assay, respectively.

2.4. Statistical analysis

The results are presented as means (± SD). All given parameters were compared between the CONTR group and the LPS group using repeated measures analysis of variance (ANOVA). In this model, the effect of the group and time p.c., as well as the interaction of group and time p.c. were assessed. Differences were considered significant at $P < 0.05$. All analyses were performed in SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA).

3. Results

The eventual number of animals in the LPS group was reduced from 11 to 9. The clinical condition at 0 h led to the exclusion of one calf, due to the presence of fever (40.7 °C). The second calf was euthanized 50 min p.c. because of the appearance of severe shock symptoms. The necropsy and histological examination revealed acute diffuse alveolar damage, as well as compensatory changes of the myocardium. More specifically, the lung lesions included general congestion and oedema, multiple haemorrhages, multifocal alveolar emphysema and hyaline membrane formation. Infiltration of macrophages and neutrophils in the alveolar septa was observed, while inter-alveolar macrophages and neutrophils were less frequent. The myocardial changes, on the other hand, were indicative of an earlier injury, and might have enhanced the sensitivity to LPS.

3.1. Clinical signs

The CONTR group showed no changes in rectal body temperature, respiratory rate and heart rate during the experiment, with mean values being 38.5 ± 0.2 °C, 30 ± 3 bpm and 73 ± 5 bpm, respectively. Following the LPS challenge, all parameters altered significantly, whereas normalization was established within 24 h p.c. (Fig. 1.1-1.2). The mean rectal body temperature increased gradually in the LPS group and reached a maximum of 39.4 °C at 4-5 h p.c., after which a slow decrease was initiated (Fig. 1.1; Table 1.1). However, two different visual trends were observed regarding the course of the rectal body temperature in this group: five calves showed an early rise in rectal body temperature (≤ 5 h p.c.), whereas the remaining four calves presented a more delayed development of fever (≥ 6 h p.c.), which was preceded by a slight drop in the body temperature at 3h p.c. (Fig. 1.1). The respiratory rate rose sharply in all calves after LPS administration, reaching a peak at 0.5 h p.c. (92 ± 24 bpm), followed by a rather fast decline in the next hour (Fig. 1.2A). Regarding the heart rate in the LPS group, a subdivision was made, based on the different rectal body temperature trends (Fig. 1.2B). In the early responding rectal body temperature group (n = 5), the heart rate demonstrated a quite similar profile as for rectal body temperature, with a maximum at 5 h p.c. (118 ± 12 bpm). The delayed responding rectal body temperature group (n = 4), on the other hand, showed a biphasic heart rate course, reaching a first peak at 3.5 h p.c. and a second peak at 8 h p.c.

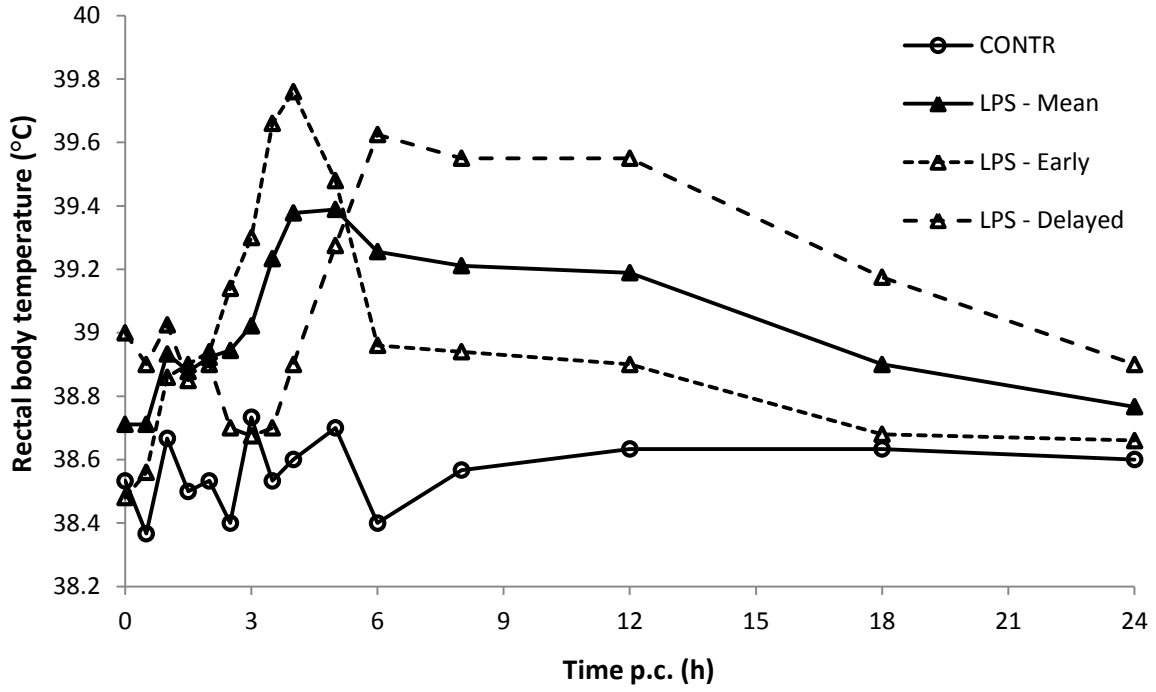

Figure 1.1. Time course of the mean rectal body temperature following an IV bolus injection of either 0.5 µg/kg BW lipopolysaccharide (LPS; n = 9) (full line, closed triangle) or a similar volume of saline (CONTR; n = 3) (full line, open circle). The dash lines (open triangles) result from the subdivision of the calves in the LPS group in an early (n = 5) (short dashes) and a delayed responding group (n = 4) (long dashes), based on the time of maximal rectal body temperature (≤ 5 h or ≥ 6 h p.c.). For clarity of presentation, SDs are not included in this figure. However, Table 1.1 gives an overview of the full data set.

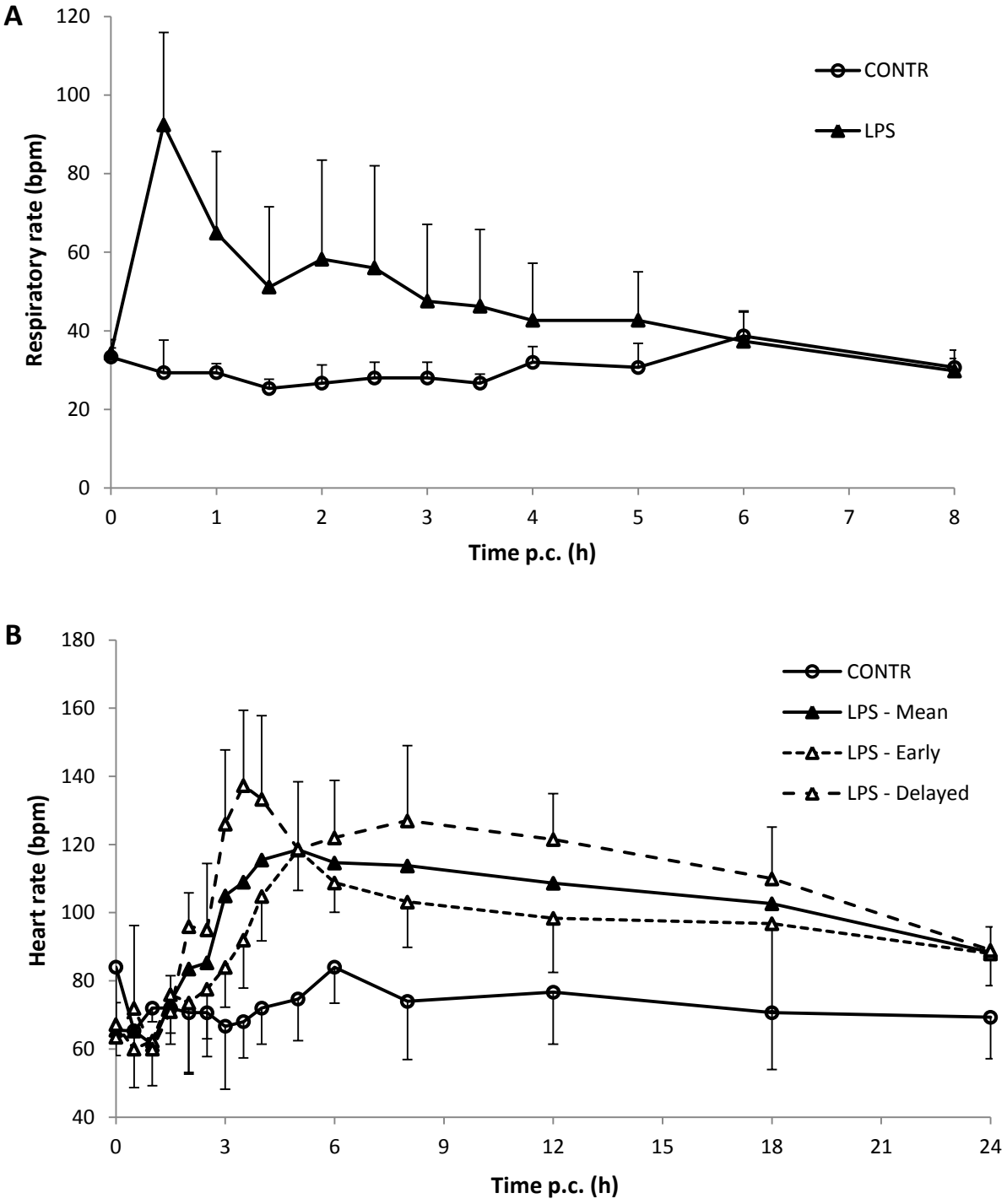

Figure 1.2. (A) Mean (+SD) respiratory rate following an IV bolus injection of either 0.5 µg/kg BW lipopolysaccharide (LPS; n = 9) (closed triangle) or a similar volume of saline (CONTR; n = 3) (open circle). (B) Mean heart rate following an IV bolus injection of either 0.5 µg/kg BW lipopolysaccharide (LPS; n = 9) (full line, closed triangle) or a similar volume of saline (CONTR; n = 3; mean (–SD)) (full line, open circle). The dash lines (open triangles) result from the subdivision of the calves in the LPS group in an early (n = 5; mean (–SD)) (short dashes) and a delayed responding group (n = 4; mean (+SD)) (long dashes) with respect to fever development (≤ 5 h or ≥ 6 h p.c.).

Table 1.1. Mean (± SD) rectal body temperature following an i.v. bolus injection of either 0.5 µg/kg BW lipopolysaccharide (LPS; n = 9) or a similar volume of saline (CONTR; n = 3). The additional subdivision of the LPS group in an early (n = 5) and a delayed responding group (n = 4) is related to the time of maximal rectal body temperature (≤ 5 h or ≥ 6 h p.c.).

Time p.c. (h)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**5**	**6**	**8**	**12**	**18**	**24**
CONTR (°C)	38.5 (0.6)	38.4 (0.4)	38.7 (0.2)	38.5 (0.3)	38.5 (0.3)	38.4 (0.2)	38.7 (0.2)	38.5 (0.4)	38.6 (0.5)	38.7 (0.3)	38.4 (0.3)	38.6 (0.4)	38.6 (0.1)	38.6 (0.3)	38.6 (0.1)
LPS Total (°C)	38.7 (0.4)	38.7 (0.3)	38.9 (0.2)	38.9 (0.3)	38.9 (0.3)	38.9 (0.4)	39.0 (0.5)	39.2 (0.6)	39.4 (0.5)	39.4 (0.2)	39.3 (0.5)	39.2 (0.5)	39.2 (0.6)	38.9 (0.4)	38.8 (0.2)
LPS Early (°C)	38.5 (0.4)	38.6 (0.2)	38.9 (0.2)	38.9 (0.2)	38.9 (0.2)	39.1 (0.2)	39.3 (0.3)	39.7 (0.4)	39.8 (0.2)	39.5 (0.1)	39.0 (0.5)	38.9 (0.5)	38.9 (0.5)	38.7 (0.3)	38.7 (0.3)
LPS Delayed (°C)	39.0 (0.3)	38.9 (0.4)	39.0 (0.2)	38.9 (0.4)	38.9 (0.5)	38.7 (0.5)	38.7 (0.4)	38.7 (0.4)	38.9 (0.4)	39.3 (0.3)	39.6 (0.1)	39.6 (0.2)	39.6 (0.3)	39.2 (0.2)	38.9 (0.1)

Regarding the clinical signs and changes in mental state, position and appetite following the LPS challenge, three behavioural phases could be identified in the first 9 h of the experiment. All calves passed through these three phases, even though the intensity and duration of each phase varied among the animals (Fig. 1.3). None of the calves showed any interest in starter mix, hay or water during the three phases. The first phase was clearly associated with LPS administration, and was initiated at 17.9 ± 3.1 min p.c. by an anxious impression of the calves and the onset of dyspnea, which was characterized by an increased respiratory effort and tachypnea (respiratory rate > 80 bpm) (respiratory phase). In addition, two calves showed open-mouth breathing, which started at 22.5 ± 3.5 min p.c. One calf, on the other hand, only showed a moderate increase in respiratory rate. The onset of respiratory distress was marked by the appearance of coughing in six out of nine calves. Stridor in consequence of dyspnea occurred in four calves, independent of the incidence of coughing. Most of the animals lay down quickly, with the mean time to lie down being 29 ± 18 min p.c. The calves that remained standing after the start of respiratory distress adopted a pose with extended neck and lowered head (n = 3). Most animals lay in sternal position, with their head and neck contacting the floor, whereas two of the calves demonstrated lateral decubitus immediately after lying down. None of the calves showed any interest in the environment during this phase, which continued up to the moment of recovery from respiratory distress (1.75 ± 0.9 h p.c.). The second phase was characterized by moderate depression that deteriorated again while fever developed (depression phase). Six calves showed lateral decubitus over a certain period in this phase. Finally, the last phase was distinguished by general recovery of the animals and could be recognized by regained alertness and vitality (recovery phase). Depression diminished after 5.0 ± 1.9 h, with adoption of a sternal position. The mean time at which the calves got up for the first time after the LPS challenge was 6.1 ± 1.1 h p.c., and was regarded as an indication of full recovery. Most calves drank water shortly after standing up. Milk (1 L) was first presented 7 h p.c., and was refused by one calf at that moment. At 13 h p.c., none of the calves remained anorexic, as the supplied 2 L of milk and a handful of starter mix were consumed easily.

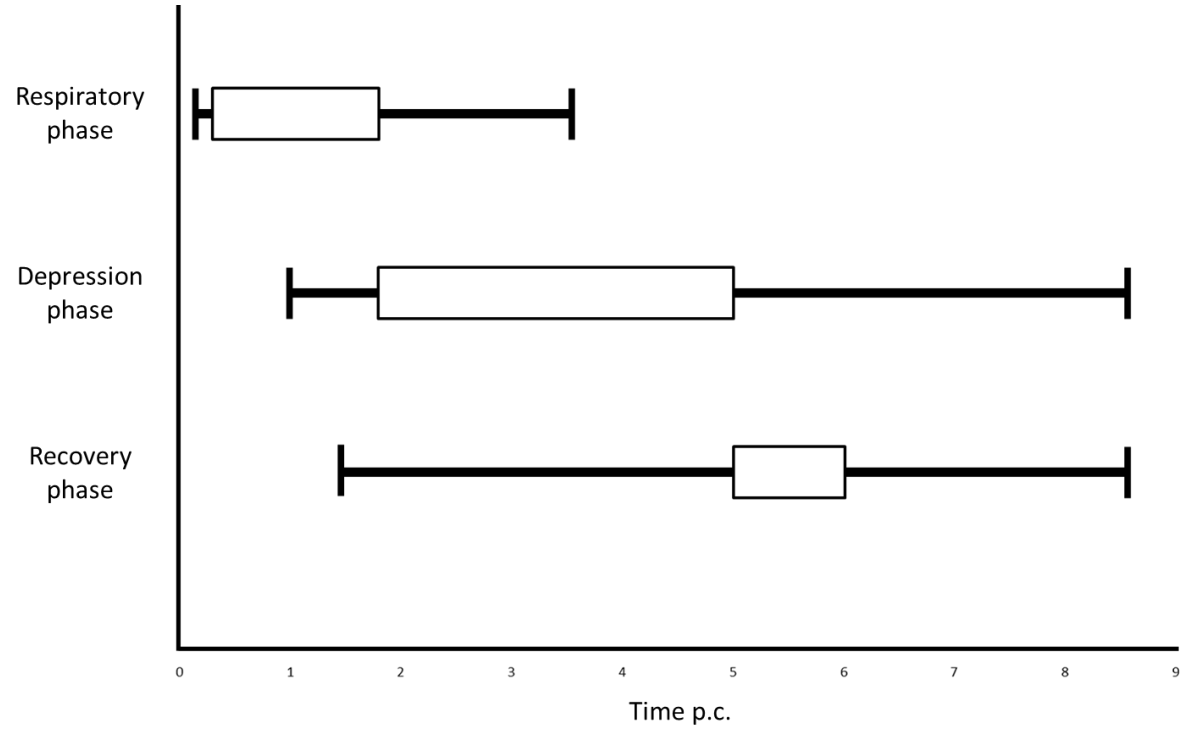

Figure 1.3. Time course of the three behavioural phases in the calves following an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide (n = 9), based on scoring of the animals' clinical condition during the first nine hours of the experiment. The boxes present the mean duration of the different phases, whereas the vertical lines indicate the minimum and maximum points in time at which the respective phase starts and ends, within a certain calf. The start of the respiratory phase is characterized by the onset of dyspnea. Recovery from respiratory distress, on the other hand, displays the end of this first phase, and simultaneously indicates the start of the depression phase. The latter ends when alertness is regained, while this observation initiates the recovery phase. The time at which the calf stands up is referred to as the end of this last phase, and consequently indicates full recovery of the calf.

3.2. Inflammatory mediators

As can be observed from Fig. 1.4 and 1.5, the CONTR group presented no detectable or only basal concentrations of TNF-α, IL-6, SAA and Hp during the course of the experiment. Following the LPS challenge, on the other hand, plasma concentrations of these inflammatory mediators increased significantly. Cytokine levels normalized within 8 h p.c., whereas the acute-phase proteins returned to pre-challenge concentrations within 54 h p.c. TNF-α levels increased dramatically in the LPS group, reaching a peak as soon as 1 h p.c. (Fig. 1.4A). Maximal IL-6 concentrations were recorded at a later time, namely at 3.5 h p.c. (Fig. 1.4B). For IL-1β, on the other hand, only very low concentrations were measured in the LPS group following a 1/2 plasma dilution. However, as the validation procedure revealed a minimal dilution factor of 1/5 for plasma, no IL-1β levels could be demonstrated following LPS administration. As shown in Fig. 1.5A, the LPS group reached peak SAA plasma concentrations at 24 h p.c. Maximal Hp levels were determined prior to the SAA peak, namely at 18 h p.c. (Fig. 1.5B).

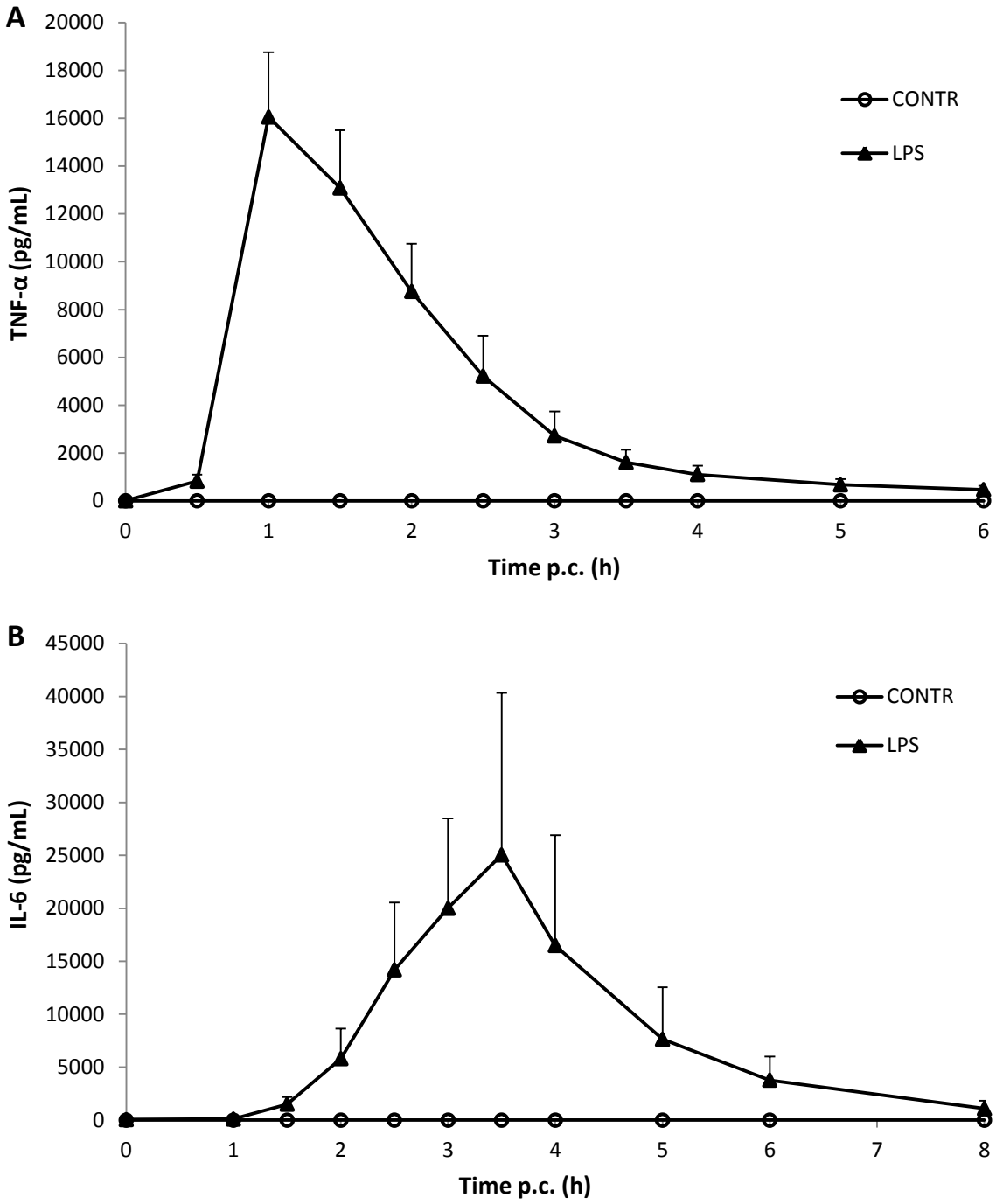

Figure 1.4. Mean (+SD) plasma concentrations of TNF-α (A) and IL-6 (B) following an IV bolus injection of either 0.5 µg/kg BW lipopolysaccharide (LPS; n = 9) (closed triangle) or a similar volume of saline (CONTR; n = 3) (open circle).

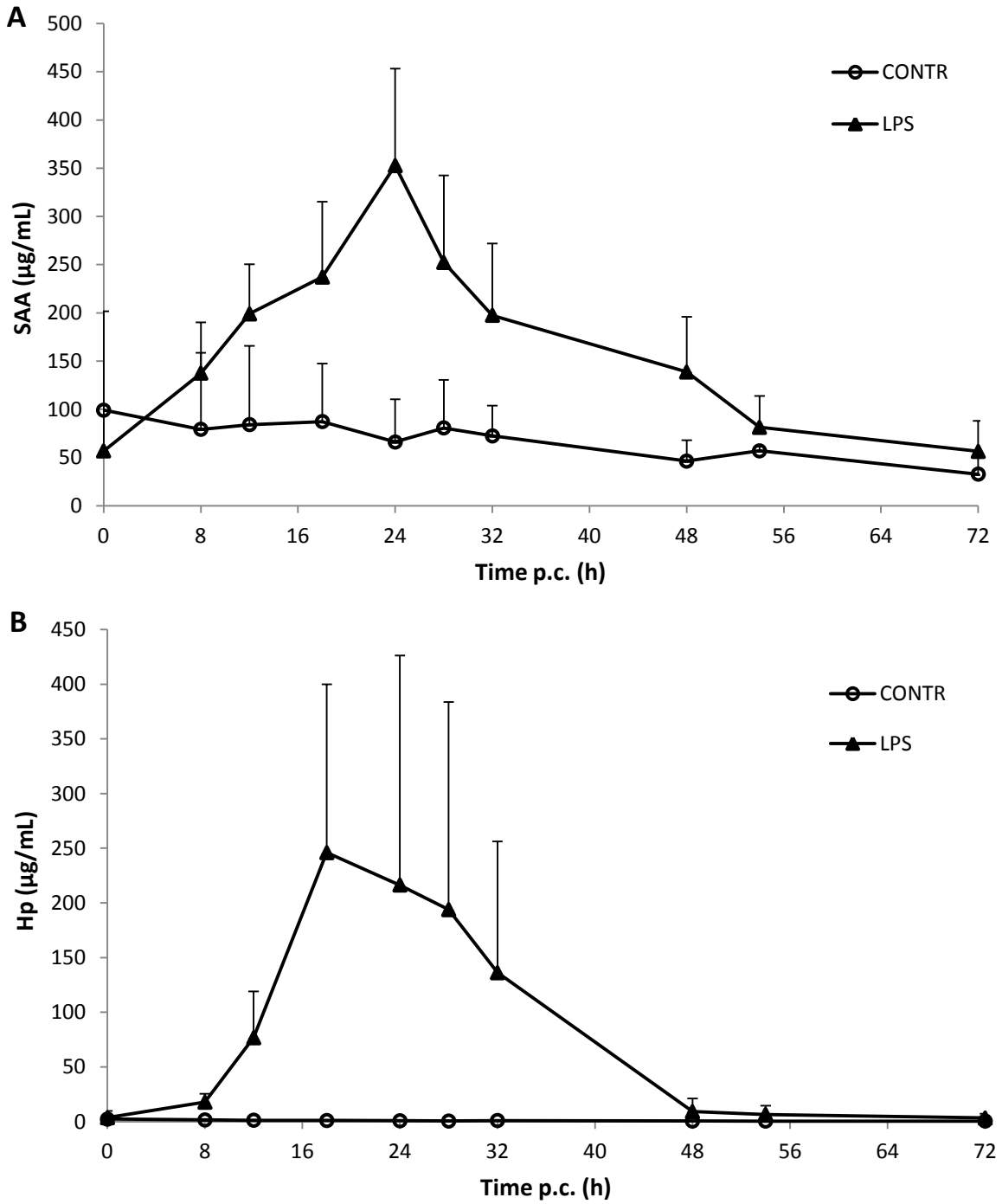

Figure 1.5. Mean (+SD) plasma concentrations of SAA (A) and Hp (B) following an IV bolus injection of either 0.5 µg/kg BW lipopolysaccharide (LPS; n = 9) (closed triangle) or a similar volume of saline (CONTR; n = 3) (open circle).

4. Discussion

The present study demonstrates the feasibility of this LPS inflammation model to mimic the acute-phase response in calves. Besides a significant alteration of cytokine and acute-phase protein concentrations, non-specific symptoms of sickness were induced, including fever and depression. Furthermore, this is the first inflammation model in calves (< 6 months) reporting increased plasma concentrations of IL-6, as well as of both SAA and Hp. The results of this study can serve as a basis to define the immunomodulatory properties of (N)SAIDs and antimicrobial drugs at different levels of the acute-phase response.

Several research groups use neonatal calves for their experiments, which are obtained from local farms within 6 h of birth (Adams *et al.*, 1990; Gerros *et al.*, 1993; Rose and Semrad, 1992; Semrad *et al.*, 1993). Upon arrival at the experimental site, these colostrum-deprived calves receive pooled colostrum. Calves with negative results of a faecal screening test for K-99 antigen and positive results of a zinc sulphate turbidity test for colostral antibodies subsequently enter the study between 24 and 36 hours of age. Bieniek *et al.* (1998), on the other hand, obtained calves from local farms that were raised for a minimum of 19 days with their mother. Given the fact the mentioned studies in neonatal calves are not straightforward with respect to fever development, the present study applied a method of animal collection quite similar to that of Bieniek *et al.* (1998).

Although calves are less sensitive to handling stress than for example pigs, three control animals were included in the present study. Stress can induce elevated cortisol concentrations, which in turn can result in the release of IL-6 (Cooke and Bohnert, 2011). Nevertheless, the calves in the present study were habituated to human presence and contact, as well as to experimental manipulations during the acclimatisation period. The indwelling catheter for blood collection was an additional measure to reduce stress. Indeed, the CONTR animals demonstrated no changes in their clinical condition, nor in cytokine or acute-phase protein concentrations. Therefore, we agree with the statement of Carroll *et al.* (2009b) that control treatments may not be necessary in these kind of experiments. Instead, animal-specific pre-challenge values could serve as an adequate control. For this reason, repeated measures ANOVA was selected as statistical test.

LPS exerts its effect through binding to Toll-like receptor 4 (TLR4), expressed on the surface of innate immune cells. As a consequence, an intracellular signaling pathway is initiated, which ultimately results in the activation of NF-κB. The latter stimulates the transcription of genes coding for pro-inflammatory cytokines and mediators, which play a key role in triggering the acute-phase response. The generally applied *E. coli* serotypes in bovine endotoxemia studies are O55:B5 and O111:B4. Since reports on the development of fever were not straightforward following the administration of serotype O55:B5, we selected the O111:B4 serotype for the present study (Kenison *et al.*, 1991; Rose and Semrad, 1992). Additionally, Carroll *et al.* (2009b) demonstrated that this serotype resulted in the onset of fever, as well as in elevated concentrations of all studied pro-inflammatory cytokines and SAA in steers. In contrast with previous studies using the O111:B4 serotype, we administered a highly purified and well-characterized LPS batch. It was confirmed by the manufacturer that this type of ultrapure LPS only activates the TLR4 pathway. Most LPS preparations, on the other hand, are contaminated by other bacterial components, in this way activating both TLR4 and TLR2 signaling (Rutledge *et al.*, 2012). The applied dose of 0.5 µg/kg BW (500 units/kg BW) was carefully selected based on the different doses used in the studies of Werling *et al.* (1996), Bieniek *et al.* (1998) and Carroll *et al.* (2009b), in heifers, calves and steers, respectively. Since calves are physiologically extremely sensitive to LPS, the doses used in older animals were thought to be too high (Michaels and Banks, 1988). Bieniek *et al.* (1998), on the other hand, described their dose of 0.1 µg/kg BW to be low. For this reason, an intermediate amount of endotoxin was applied in the present study. Except for occurrence of shock symptoms in one animal, this was indeed found to be a suitable dose with respect to the aims of our research. The postulated exclusion criterion (rectal body temperature ≥ 40 °C), on the other hand, might have been too high. Therefore, in our opinion, this criterion should be adapted to at least 39.5 °C for future experiments.

TNF-α has been put forward to be the principal mediator of acute inflammation in response to Gram-negative bacteria (Conti *et al.*, 2004). The profile of our TNF-α plasma concentration-time curve showed a marked similarity to previously reported experiments: a sharp increase with a peak at 1 h p.c., followed by a rather fast decline, and a nearly complete normalization by 4 h p.c. The early peak was confirmed by previous endotoxin challenges in young calves (< 6 months) (Adams *et al.*, 1990; Gerros *et al.*, 1993; Kinsbergen

et al., 1994), although other authors reported maximal levels at 2 h p.c. (Kenison *et al.*, 1991; Semrad *et al.*, 1993; Bieniek *et al.*, 1998; Kushibiki *et al.*, 2008). Nevertheless, in the study of Kushibiki *et al.* (2008) blood sampling at 1 h p.c. was not included in the experimental design. Furthermore, no influence of age, the administered dose or serotype or method of administration (bolus or infusion) was observed. Plasma concentrations of IL-1β were very low in the LPS group of our experiment and could not be quantified due to the required dilution factor. In contrast to the frequent determination of TNF-α, only one study in calves reported increased activity of IL-1β in response to an endotoxin challenge, with maximal values at 3-3.5 h p.c. (Gerros *et al.*, 1993). Research in older animals demonstrated maximal, yet low plasma concentrations of IL-1β at 3 h p.c. as well (Carroll *et al.*, 2009b). These findings might be attributed to the rather low efficiency of IL-1β mRNA translation (Bailly *et al.*, 1994). The present study is the first to report IL-6 levels in LPS-treated calves. Maximal concentrations were observed 3.5 h p.c., which is considerably later than the peak of TNF-α. This is in accordance to the findings of Carroll *et al.* (2009b) in steers, with a recorded peak of IL-6 at 3 h p.c. In our research, this cytokine's plasma concentration-time curve had a rather symmetric shape, indicating a more gradual increase and decrease in comparison with TNF-α. Carroll *et al.* (2009b), on the other hand, still detected high levels of IL-6 at their final sampling time (8 h p.c.).

Once released, these pro-inflammatory cytokines initiate the acute-phase response in collaboration with other peripheral inflammatory mediators, such as arachidonic acid metabolites, ROS and NO. Interestingly, the first clinical symptoms following LPS administration (17.9 ± 3.1 min p.c.), including dyspnea, coughing and stridor, preceded the increasing levels of pro-inflammatory cytokines. This early onset of the respiratory phase might be explained by the role of the lung as the major target organ for endotoxin in cattle, rapidly resulting in an increased respiratory drive, dyspnea, diffuse pulmonary oedema and congestion, as well as airway haemorrhages (Tikoff *et al.*, 1966; Ohtsuka *et al.*, 1997b; Preas *et al.*, 2001). These lesions were confirmed by the histological examination of the lungs of the euthanized calf in our experiment at 50 min p.c. The open-mouth breathing, observed in two of the calves in our experiment, reflects the occurrence of severe respiratory distress and hypoxemia (Constable *et al.*, 1991).

Reports on the course of the respiratory rate following LPS challenges in calves are not straightforward. In our experiment, the respiratory rate clearly peaked during the respiratory phase, with a maximal rate determined at the first sampling point (0.5 h p.c.). This early peak was confirmed by Constable *et al.* (1991) and Bieniek *et al.* (1998), while Borderas *et al.* (2008) and Kinsbergen *et al.* (1994) observed maximal frequencies only 3 h and 4 h p.c., respectively. Rose and Semrad (1992), on the other hand, reported no significant increase in respiratory rate following experimental endotoxemia in neonatal calves. In this respect, differences related to the administered dose, method of administration and age of the calves have been suggested to be of importance (Bieniek *et al.*, 1998; Borderas *et al.*, 2008). However, no trend could be observed depending on these factors, nor an effect of the specific LPS serotype selected for the challenge.

During the respiratory phase, the calves were severely depressed, although respiratory symptoms predominated. The depression phase, on the other hand, resembled more to the development of naturally occurring illness, with anorexia and deteriorating depression while fever developed. In this regard, cytokines (particularly IL-1β and TNF-α) and prostaglandins play a pivotal role, inducing non-specific symptoms of illness following an LPS challenge (Johnson, 2002; Conti *et al.*, 2004; Dantzer; 2009; Pecchi *et al.*, 2009). Simultaneously with the induction of sickness behaviour, IL-1β and IL-6 stimulate the hypothalamic-pituitary-adrenal axis and the subsequent production of adrenocorticotropic hormone and glucocorticoids (Werling *et al.*, 1996; Carroll *et al.*, 2009b). As a result, further cytokine gene-expression is inhibited through a negative feedback mechanism (Baybutt and Holsboer, 1990; Turnbull and Rivier, 1999).

The onset of fever might be the most frequently studied effect following an LPS challenge. Both pyrogenic cytokines (TNF-α, IL-1β and IL-6 being the most relevant) and PGE2 have been reported to be of importance in fever development (Godson *et al.*, 1995; Kushibiki *et al.*, 2000; Conti *et al.* 2004; Blatteis, 2007). Also in the present study, a distinct effect of endotoxin on the body temperature was observed in calves. The occurrence of lateral decubitus during this febrile response is thought to be a form of thermoregulatory behaviour, increasing heat loss (Borderas *et al.*, 2008). The rectal body temperature increased in our experiment from 1 h and up to 4-5 h p.c., which fairly corresponds to the

observations of Semrad (1993a), Kinsbergen *et al.* (1994), Bieniek *et al.* (1998) and Borderas *et al.* (2008) in young calves. Kenison *et al.* (1991), on the other hand, did not attain a well-defined fever response in 3-month-old calves. In this respect, the authors suggested that the applied dose of 1 µg/kg BW of LPS might have been too low. Rose and Semrad (1992) reported a non-responding rectal body temperature as well following a 3-h-lasting IV endotoxin infusion of 3.25 µg/kg BW in neonatal calves. Nevertheless, the lowest dose studied in calves (0.025 µg/kg BW) already resulted in a marked increase in rectal body temperature (Borderas *et al.*, 2008). The age of the calves in the latter study (3 weeks old) might have been of importance, although calves are considered immunocompetent at birth (Buddle *et al.*, 2003). The degree of fever development is difficult to compare between different experiments, as many factors can interfere, including the administered dose, method of administration, method of body temperature determination, environmental temperature and breed (Johnson and von Borell, 1994; Carroll *et al.*, 2013). Concerning the effect of the administered dose, Jacobsen *et al.* (2005) made a remarkable conclusion in adult cows: increasing doses of LPS (10 – 100 – 1000 ng/kg BW) resulted in a mono-, bi-, and triphasic pattern of the rectal body temperature, respectively. Different "waves" of PGE2 synthesis might be of importance here (Blatteis *et al.*, 2005; Blatteis, 2007). Furthermore, these authors reported an inverse relationship between the dose of endotoxin and the rectal body temperature, which could be attributed to a pre-shock effect in response to higher doses (Lohuis *et al.*, 1988). This pre-shock effect might be an explanation for the two different rectal body temperature trends observed in our experiment. More specifically, the delayed responding rectal body temperature group showed a slight decline in rectal body temperature shortly after the LPS-challenge (2.5 h p.c.), whereas the early responding rectal body temperature group presented increasing values at this time. In this respect, we can conclude that individual variations in LPS-induced responses cannot be neglected. Several devices have been used in cattle to determine the body temperature. The rectal application of a digital thermometer is overall the most frequently reported method (Werling *et al.*, 1996; Borderas *et al.*, 2008). Nevertheless, the accuracy of this measurement can be influenced by several factors, including defecation and repeated insertions of the thermometer (Burfeind *et al.*, 2013). The use of an automatic rectal body temperature monitoring device might therefore be an interesting solution (Carroll *et al.*, 2009b; Reuter *et*

al., 2010). Besides the decreased labour for researchers and the absence of handling stress in the animals, this device is able to record at 1 minute intervals. However, its suitability for rectal body temperature determination in young calves has not been evaluated. Carroll *et al.* (2009b) also determined the skin surface temperature in their endotoxin experiment. More specifically, shaved surfaces of the shoulder, rump and ear were recorded using an infrared thermometer. Although skin temperatures at the shoulder and rump increased following the endotoxin challenge, this was not as closely related to the LPS challenge as the rectal body temperature. Moreover, the ear skin temperature decreased dramatically during the experiment. Another method to evaluate the body temperature in older animals is by a temperature-sensitive bolus, which is placed in the rumen (Carroll *et al.*, 2009b). Still, Reuter *et al.* (2010) reported that the LPS challenge affected the rectal body temperature more significantly than the ruminal temperature.

Simultaneously with the onset of fever, the heart rate increased in the present study. The first peak in the biphasic heart rate profile of the delayed responding rectal body temperature group (3.5 h p.c.) might be attributed to the previously mentioned pre-shock effect. As for the respiratory rate, reports on the course of the heart rate from other experiments are not completely straightforward. Kinsbergen *et al.* (1994) and Bieniek *et al.* (1998) both confirm our findings, with nearly coinciding maxima for the heart rate and rectal body temperature. Kenison *et al.* (1991), on the other hand, recorded rapid heart rates already during the initial reaction to endotoxin. Interestingly, Borderas *et al.* (2008) reported no differences between HRs in LPS and saline challenged calves, not even around the time of maximum rectal body temperature.

The acute-phase response is generally regarded to be beneficial to the organism, as it is important for providing protection to the animal, as well as for initiating events to restore the animal to a homeostatic condition (Carroll *et al.*, 2009b). Nevertheless, when the reaction becomes dysregulated or excessive, it can result in life-threatening syndromes, such as sepsis and septic shock (Lillie, 1974; Peri at al., 2010). The slight decline in rectal body temperature shortly after the LPS challenge in the delayed responding group was previously linked to a pre-shock effect (Lillie, 1974). In human medicine, it has been described that hypothermic patients with septic shock have a significantly higher mortality than febrile

septic shock patients (Marik and Zaloga, 2000). Indeed, the calves in the early responding rectal body temperature group generally recovered faster from the challenge in comparison with the animals in the delayed responding group. Concerning the occurrence of shock, TNF-α has been reported to be an important mediator (Beutler, 1985). In this respect, the over-stimulation of TNF-α production by large amounts of endotoxin may result in intravascular coagulation, hypotension and shock (Rietschel and Brade, 1992). NO, which is released from endothelial cells immediately following an endotoxin challenge, contributes to shock development as well (Salvemini *et al.*, 1990). During sepsis, both TNF-α and NO induce vascular dilatation, causing a large decrease in systemic vascular resistance. This situation can lead to distributive or vasodilatory (septic) shock, characterized by a hyperdynamic phase with increased heart rate and cardiac ouput (Klosterhalfen *et al.*, 1992). This early hyperdynamic phase can evolve to a late hypodynamic phase, with a low cardiac output and a high systemic vascular resistance, accompanied by cold extremities (Garrido *et al.*, 2004; Carroll *et al.*, 2009b). Nevertheless, as mentioned before, endotoxin models do not accurately mimic patients with sepsis (Remick and Ward, 2005). Septic shock, for example, is often the result of vascular invasion by bacteria, which is consequently lacking following an LPS challenge (Fink and Heard, 1990). In this respect, Garrido *et al.* (2004) described in their review article that lower doses of LPS seem to result in the hyperdynamic phase of shock, whereas larger endotoxin doses induce the hypodynamic phase, resulting in rapid circulatory collapse and death. From this point of view, we can state that the dose of LPS applied in the present study rather induced a hypodynamic circulatory pattern. More specifically, the shock symptoms in the euthanized calf included tachycardia, tachypnea, lateral decubitus, pale mucosae, slow capillary refill and cold extremities.

Besides the occurrence of non-specific symptoms of sickness, the acute-phase response is characterized by the hepatic synthesis of acute-phase proteins. Our LPS challenge induced increasing levels of both SAA and Hp, reaching maximal plasma concentrations at 24 and 18 h p.c., respectively. These findings emphasise the importance of a prolonged sampling period following an endotoxin challenge with respect to the study of acute-phase proteins. Reports on acute-phase proteins following experimental endotoxemia are clearly less frequent in calves in comparison with reports on cytokines. Kushibiki *et al.* (2008) reported maximal Hp levels at 24 h p.c., whereas Conner *et al.* (1989), remarkably,

observed peak Hp concentrations only 5 days p.c. In older calves (5-12 months), Elsasser *et al.* (2005) demonstrated increased levels of both acute-phase proteins following an LPS challenge. However, no conclusions on the sequence of appearance could be drawn from this study as only three sampling points were included (0, 7 and 24 h p.c.). The results of two studies in heifers and steers are rather controversial, as the sampling period only lasted for 6 and 8 h, respectively (Werling *et al.*, 1996; Carroll *et al.*, 2009b). The observations of the present study do not correspond to the conclusions of studies applying experimental infections. More specifically, the latter generally report SAA to be a more rapidly reacting acute-phase protein than Hp (Horadagoda *et al.*, 1994; Ganheim *et al.*, 2003). In this respect, Horadagoda *et al.* (1999) even concluded that SAA and Hp may be used to discriminate between acute and chronic inflammatory conditions in cattle. This discrepancy, however, might be associated with the administration of merely endotoxin in the present study.

All plasma analyses for cytokines and acute-phase proteins in the present study were performed by ELISAs. These assays are highly specific and sensitive, and are still considered as the gold standard today (Elshal and McCoy, 2006). Nevertheless, the authors strongly advise to run analyses within one specific batch, as we stated rather large differences between the results of assays from different lots for certain parameters. In this respect, the development of a multiplex assay might be of interest as well, as ELISAs are rather time and sample-consuming (Dernfalk *et al.*, 2007; Wyns *et al.*, 2013).

The clinical scoring in the present study was performed by direct observation of the animals. Borderas *et al.* (2008), on the other hand, recorded the calves before and after the LPS challenge using video cameras. Subsequently, the behaviour of the animals was scored minutely by a single observer. This enabled the authors to formulate extensive reports regarding the behaviour of the animals following experimental endotoxemia, including the duration of standing, lying sternally and lying laterally. However, such reports were not the objective of the present study. The course of the depression phase, on the other hand, was of major interest for us, as one of our aims encompassed the development of a clinical scoring system for the early detection of illness. Indeed, the early symptoms during the respiratory phase do not correspond to naturally occurring illness, but the fact that depression deteriorated while fever developed was an important finding. Once the maximal

rectal body temperature was passed in each of the calves, alertness and appetite were gradually regained. In this respect, depression among a group of calves might be indicative of a developing infection. Contradictorily, an increased respiratory rate and fever were reported to be the early signs of pneumonia in calves, while depression only developed later (Lorenz *et al.*, 2011). This statement might, however, depend on the observational skills of the animal keeper, with severe depression being secondary to fever in pneumonic calves. Accordingly, Apley (2006) emphasises the importance of detecting the earliest signs of depression with respect to the chance of success of bovine respiratory disease therapy. This author even suggests treating on the basis of depression with undifferentiated fever. To further define "depression" in calves, we can conclude from our study that anorexia and depression, characterized by sustained (lateral) decubitus, were omnipresent during the depression phase. In addition, Borderas *et al.* (2008) observed reduced self-grooming, rumination and ingestion of hay, as well as an increase in time spent lying and standing inactive. Rumination was not assessed in the present study, as only a few calves developed this behaviour during the acclimatization period. In older animals, ruminal hypomotility has indeed been established to be a good parameter for evaluating sickness behaviour (Jacobsen *et al.*, 2005).

5. Conclusion

We developed and characterized a reliable LPS inflammation model in calves, clearly inducing all aspects of the acute-phase response. In this respect, the release of pro-inflammatory cytokines and acute-phase proteins was significantly enhanced, and non-specific symptoms of sickness appeared following the endotoxin challenge. Regarding the latter, we recorded deteriorating depression while fever developed. This observation emphasises the importance of the recognition of depression in the early detection of illness.

Acknowledgements

The help of J. Goossens, J. Lambrecht, A. Osselaere, E. Russo, V. Vandenbroucke and A. Van den Bussche during the animal experiment was greatly appreciated by the authors.

CHAPTER 2

Determination of the pharmacokinetic parameters of R(–) and S(+) ketoprofen in calves following intramuscular administration of a racemic mixture

Adapted from

Plessers, E., Watteyn, A., Wyns, H., Pardon, B., De Baere, S., De Backer, P., Croubels, S., 2015. Enantioselective pharmacokinetics of ketoprofen in calves after intramuscular administration of a racemic mixture. *Journal of Veterinary Pharmacology and Therapeutics* 38, 410-413.

Abstract

The pharmacokinetic properties of ketoprofen (KETO) were determined in 4-week-old calves after intramuscular (IM) injection of a racemic mixture at a dose of 3 mg/kg body weight. Due to possible enantioselective disposition kinetics and chiral inversion, the plasma concentrations of the R(–) and S(+) enantiomer were quantified separately, using a stereospecific validated high-performance liquid chromatography (HPLC) assay with ultraviolet (UV) detection. A distinct predominance of the S(+) enantiomer was observed, as well as significantly different pharmacokinetic parameters between R(–) and S(+) KETO. More in specific, a greater value for the mean area under the plasma concentration-time curve ($AUC_{0\rightarrow\infty}$) (46.92 ± 7.75 and 11.13 ± 2.18 μg.h/mL for the S(+) and R(–) enantiomer, respectively), a lower apparent clearance (Cl/F) (32.8 ± 5.7 and 139.0 ± 25.1 mL/h.kg for the S(+) and R(–) enantiomer, respectively) and a lower apparent volume of distribution (V_d/F) (139 ± 14.7 and 496 ± 139.4 mL/kg for the S(+) and R(–) enantiomer, respectively) were calculated for the S(+) enantiomer, indicating enantioselective pharmacokinetics for KETO in calves following IM administration.

1. Introduction

KETO (Fig. 2.1) or 2-(phenyl 3-benzoyl) propionic acid is a non-steroidal anti-inflammatory drug (NSAID) that is still commonly used in a wide range of indications in human and veterinary medicine for anti-inflammatory, antipyretic and analgesic purposes (Hersh *et al.*, 2000; Lees *et al.*, 2004; Pardon *et al.*, 2012a). KETO is a chiral compound and veterinary formulations are racemic mixtures, containing both enantiomers in equal amounts. The S(+) enantiomer has been generally accepted to be pharmacologically active, through inhibition of COX (Cabré *et al.*, 1998). R(–) KETO, on the other hand, is a weak COX inhibitor, whereas the analgesic effect of KETO has been associated with this enantiomer (Cooper *et al.*, 1998; Ghezzi *et al.*, 1998).

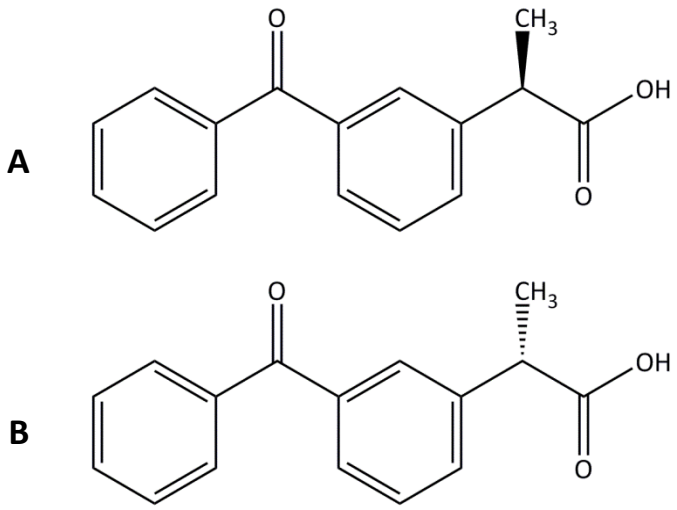

Figure 2.1. Chemical structure of R(–) ketoprofen (A) and S(+) ketoprofen (B)

As a result of the highly chiral environment of the body, the pharmacokinetic (PK) and pharmacodynamic (PD) properties can significantly differ between both KETO enantiomers. The species-specific differences in PK may be attributed to enantioselectivity in each of the pharmacokinetic processes, including chiral conversion from R(–) to S(+) KETO (Landoni and Soraci, 2001). The IV or extravascular administration of racemic KETO demonstrated enantioselective PK in sheep, camels, pigs, horses, dogs, cats and poultry, with a

predominance of the S(+) enantiomer in plasma of most species, except for sheep, camels and poultry, in which R(–) KETO predominated (Delatour *et al.*, 1993; Jaussaud *et al.*, 1993; Landoni and Lees, 1995a; Landoni *et al.*, 1999; Al Katheeri *et al.*, 2000; Arifah *et al.*, 2001; Lees *et al.*, 2003; Fosse *et al.*, 2011; Neirinckx *et al.*, 2011a; Mustonen *et al.*, 2012). In contrast, the IV administration of the racemate in calves revealed no enantioselectivity (Landoni *et al.*, 1995). Besides stereoselective PK, chiral conversion of R(–) to S(+) KETO has been confirmed in calves, cows, sheep, goats, pigs, horses, dogs, cats, rabbits and humans following the administration of R(–) KETO (Jaussaud *et al.*, 1993; Aberg *et al.*, 1995; Landoni and Lees, 1995b, 1996; Rudy *et al.*, 1998; Landoni *et al.*, 1999; Arifah *et al.*, 2001, 2003; Igarza *et al.*, 2002; Lees *et al.*, 2003; Neirinckx *et al.*, 2011b). Hence, the plasma concentrations of both enantiomers are similar in calves despite the occurrence of chiral conversion, which corresponds to the observations in goats and humans.

Notwithstanding the fact that the IM administration of KETO is highly frequent in calves, to the authors' knowledge, no data on the enantioselective behaviour of KETO following this route of administration have been reported in calves. Therefore, the aim of this study was to determine and compare the PK of the R(–) and the S(+) enantiomer after IM administration of the drug racemate. Additionally, these characteristics will be applied in further research on the immunomodulatory properties of KETO, either alone or in combination with antimicrobial drugs, in our previously developed LPS inflammation model in calves.

2. Materials and methods

Eight male 4-week-old Holstein Friesian calves, with an average BW of 53.9 ± 5.8 kg, were obtained from local farms. During an acclimatization period of one week, the animals were housed in individual pens on straw with ad libitum access to hay and fresh water. The calves were fed milk replacer three times a day, receiving a total of 5 L daily. One day before the start of the experiment, a 14G indwelling catheter (Cavafix, B. Braun, Diegem, Belgium) was placed in the jugular vein.

Racemic KETO (Ketofen® 10%, Merial, Diegem, Belgium) was administered IM in the left neck region (cervical ventral serratus muscle) at a dose of 3 mg/kg BW, as recommended by the manufacturer. Blood samples were drawn from the catheter and transferred to EDTA tubes (Vacutest Kima, Piove di Sacco, PD, Italy) just before administration and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 16, 20 and 24 h post-administration (p.a.). Blood samples were centrifuged at 1,500 x g for 15 min at 4 °C and plasma was stored at ≤ -20 °C until analysis, for a maximum of 2 months. All procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University) (EC 2013/28).

Plasma concentrations of R(–) and S(+) KETO were quantified by a validated HPLC-UV method, preceded by derivatization of both enantiomers. Both the racemic KETO standard and the racemic internal standard, fenoprofen, were obtained from Sigma-Aldrich. First, plasma samples (500 µL) were spiked with internal standard, acidified using 200 µL HCl (1 mol/L), extracted with 5 mL of isooctane-isopropanol (95:5, v/v), and subsequently, the organic phase was evaporated under a stream of nitrogen at 40 °C. The derivatization of KETO and fenoprofen was then performed using L-leucinamide, principally according to the method described by Foster and Jamali (1987). L-leucinamide converts the enantiomers into their diastereomers, which potentiates their separation on a conventional HPLC reversed-phase column. Subsequently, a 20 µL aliquot of the sample was injected onto a Thermo Separation Products (Fremont, CA, USA) HPLC system consisting of a P-4000 pump, an AS 3000 autosampler and a Spectra Focus Forward scanning UV-detector set at 260 nm. The R(–) fenoprofen peak was used as internal standard.

The PK parameters were calculated by means of the WinNonlin software program, version 6.3 (Pharsight Corporation, Mountain View, CA, USA), using a non-compartmental and a one-compartmental method for R(–) and S(+) KETO, respectively. The model with the best fit was selected after visual inspection of the fitted curve. Values below the limit of quantification of 0.05 µg/mL were excluded from the PK analysis. The area under the plasma concentration-time curve ($AUC_{0\rightarrow\infty}$) was calculated using the trapezoidal method with extrapolation to infinity. The elimination rate constant (λz) was deduced from the lower part of the plasma concentration-time curve for the R(–) enantiomer. Data are expressed as means ± standard deviation (SD) and were statistically analysed by means of a Mann-

Whitney U test, using SPSS 21.0 software for Windows (IBM Corp., Armonk, NY, USA). Differences were considered significant at $P < 0.05$.

3. Results

Semi-logarithmic plots of the mean R(–) and S(+) KETO plasma concentration versus time curves after IM administration of the racemic mixture are presented in Figure 2.2. Quantifiable concentrations of R(–) and S(+) KETO were measured until 10 h and 20 h p.a., respectively. The mean calculated PK parameters for both enantiomers are summarized in Table 2.1, indicating that most parameters differ significantly between R(–) and S(+) KETO.

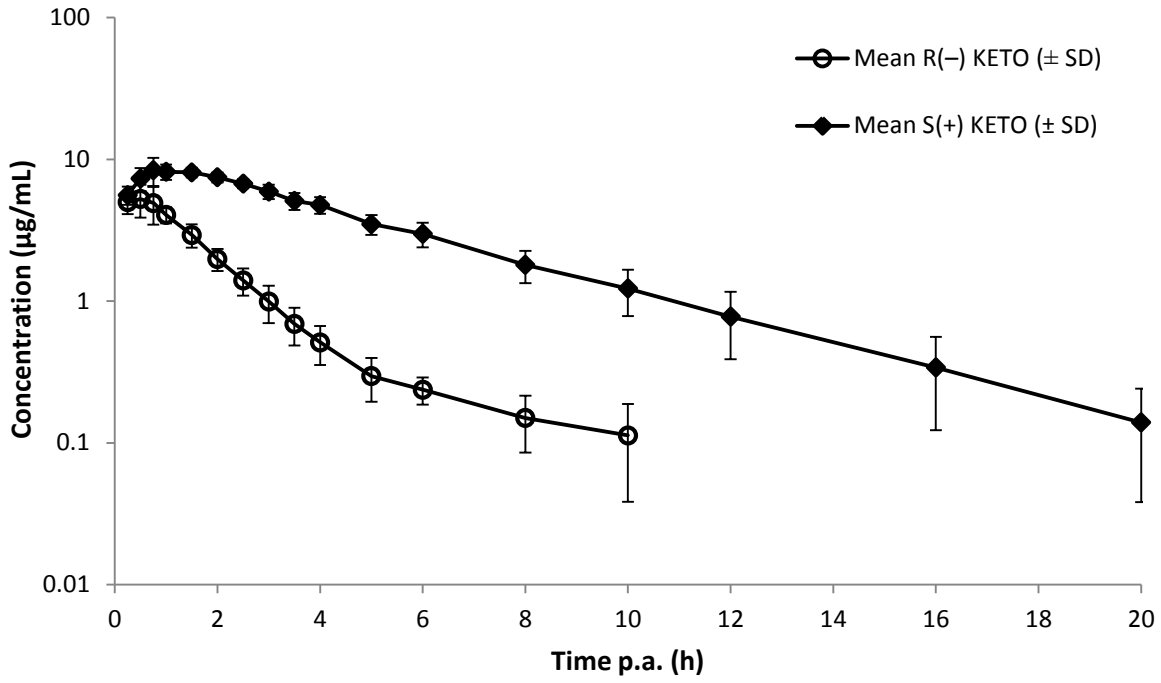

Figure 2.2. Mean (± SD) plasma concentration-time profiles of R(–) and (S+) KETO after a single IM administration of 3 mg/kg BW of the racemic mixture in calves (n = 8).

Table 2.1. Pharmacokinetic parameters (mean ± SD) of R(–) and S(+) KETO in calves after a single IM administration of 3 mg/kg BW of the racemic mixture, using a non-compartmental and a one-compartmental method for R(–) and S(+) KETO, respectively (n = 8).

Parameter	Units	R(–) KETO	S(+) KETO
$AUC_{0\rightarrow\infty}$	µg.h/mL	11.13 ± 2.18[a]	46.92 ± 7.75[b]
k_{el}	/h	0.29 ± 0.06	0.24 ± 0.05
$t_{1/2el}$	h	2.45 ± 0.48	3.02 ± 0.60
t_{max}	h	0.50 ± 0.23[a]	0.98 ± 0.18[b]
C_{max}	µg/mL	5.40 ± 1.41[a]	8.56 ± 0.80[b]
Cl/F	mL/h.kg	139.0 ± 25.1[a]	32.8 ± 5.7[b]
V_d/F	mL/kg	496 ± 139.4[a]	139 ± 14.7[b]
MRT	h	2.30 ± 0.33	–

$AUC_{0\rightarrow\infty}$, area under the plasma concentration-time curve, extrapolated to infinity; k_{el}, elimination rate constant; $t_{1/2el}$, half-life of elimination; t_{max}, time to maximum plasma concentration; C_{max}, maximum plasma concentration; Cl/F, total clearance not corrected for bioavailability; V_d/F, volume of distribution not corrected for bioavailability; MRT, mean residence time.
Different letters within a row indicate significant differences between the enantiomers.

4. Discussion

The present study is the first to describe the PK of R(–) and S(+) KETO after IM administration of a racemic mixture in calves. Similar to most of the animal species mentioned above, a predominance of the S(+) enantiomer was determined at all sampling points. In this context, a lower apparent clearance (Cl/F) and a greater $AUC_{0\rightarrow\infty}$ value were calculated for this enantiomer, as well as a lower apparent volume of distribution (V_d/F). However, the identification of the processes involved in the observed enantioselectivity could not be determined due to the experimental design.

In calves, only the chiral conversion metabolic phenomenon, which occurs predominantly in the liver, has been reported as a factor possibly contributing to stereoselective PK (Mehvar and Jamali, 1988; Landoni and Lees, 1995b; Igarza *et al.*, 2002). The extent of chiral conversion is highly species specific, ranging from 5% in sheep to 51% in

newborn calves (Landoni *et al.*, 1999; Igarza *et al.*, 2002). However, as mentioned earlier, enantioselectivity in each of the pharmacokinetic processes can also be involved. As drugs are generally absorbed through passive non-ionic diffusion, and absorption by active processes is not reported for KETO, stereospecificity is not expected in this phase. Differences between the enantiomers regarding their distribution, on the other hand, can be the result of enantioselective binding to plasma or tissues proteins (Tucker and Lennard, 1990). As with other NSAIDs, KETO is very highly bound to plasma proteins, resulting in low V_d values (V_d/F in the present study was 496 ± 139.4 mL/kg and 139 ± 14.7 mL/kg for R(–) and S(+) KETO, respectively) (Lees *et al.*, 2004). In cattle, 97% protein binding has been reported for KETO, while data on binding of the respective enantiomers in calves are lacking (EMA, 1995). Still, the extent of protein binding differed significantly between the enantiomers in female camels and humans, although drug and protein concentrations as well as differences in protein configuration might be of importance (Dubois *et al.*, 1993; Al Katheeri *et al.*, 2000). The higher plasma protein binding of R(–) KETO compared to S(+) KETO in camels was reflected by both a higher V_d and a higher Cl for the S(+) enantiomer. However, a higher Cl for one enantiomer could also be attributed to enantioselectivity in elimination. For example, stereoselective glucuronidation has been stated for KETO in rabbits, which partially contributed to the faster clearance of the R(–) enantiomer (Abas and Meffin, 1987). *In vitro* studies using canine liver microsomes, on the other hand, demonstrated a formation ratio of S(+) to R(–)-glucuronides of 4.5 (Chakir *et al.*, 1994). Besides this enantioselectivity in biotransformation, biliary and urinary excretion have been reported to be stereoselective as well. A preferential biliary excretion of the S(+) enantiomer in rats, followed by an enantioselective enterohepatic circulation in favour of S(+) KETO, resulted in a significantly longer half-life of elimination ($t_{1/2el}$) for this enantiomer (Menzel *et al.*, 1993). In addition, Foster *et al.* (1988) recorded a higher recovery of S(+) KETO in urine of humans after the administration of a racemic mixture. Stereoselectivity in the renal processes of active secretion and, yet less likely, active reabsorption have been suggested in this context (Tucker and Lennard, 1990).

The present study demonstrated significant differences between the enantiomers regarding the maximum plasma concentration (C_{max}) and the time to maximum plasma concentration (t_{max}). These two parameters have been reported to be indirect measures of

rate of drug absorption, although their reliability has been questioned repeatedly (Endrenyi *et al.*, 1991; Lacey *et al.*, 1994). In this respect, Lacey *et al.* (1994) established that $C_{max}/AUC_{0\rightarrow\infty}$ is a more sensitive and powerful indirect measure for estimation of absorption rate. The application of this metric on our data revealed a ratio of 0.48 ± 0.05 and 0.19 ± 0.02 for R(–) and S(+) KETO, respectively, indeed reflecting a significant difference in rate of absorption between the enantiomers. Accordingly, a PK study following IM administration of the enantiomers individually could further elucidate this observation, and could reveal whether chiral inversion is a contributing factor.

In contrast to the results of the present study in 4-week-old calves, enantioselective PK of KETO were not reported after IV administration of the same dose of the racemic mixture in 20-week-old ruminating calves, with a mean BW of 119 kg (Landoni *et al.*, 1995). These authors observed nearly identical values for AUC and Cl for both enantiomers. Moreover, after administration of this 3 mg/kg BW dose, the values for $AUC_{0\rightarrow\infty}$ and half-life of elimination ($t_{1/2el}$) were found to be remarkably higher in the present study using IM administration in 4-week-old calves compared to IV administration in 20-week-old calves (for S(+) KETO, 46.92 and 9.94 µg.h/mL for AUC and 3.02 and 0.42 h for $t_{1/2el}$, respectively) (Landoni *et al.*, 1995). This finding can be explained by a ten-fold lower Cl/F in 4-week-old calves in comparison with 20-week-old calves (for S(+) KETO, 32.8 and 330 mL/h.kg, respectively). As glucuronidation to an acyl-glucuronide has been reported to be the major biotransformation process in most animal species, the lower Cl/F might be the result of an immature hepatic capacity for metabolizing drugs in young, mainly milk-fed calves (Shoaf *et al.*, 1987; Kawalek and El Said, 1994; Mauleón *et al.*, 1996; Igarza *et al.*, 2004).

Unlike the observations of Landoni *et al.* (1995), the IV administration of the enantiomers individually demonstrated enantioselective differences in ruminating calves weighing on average 113 kg (Landoni and Lees, 1995b), as well as in newborn calves (Igarza *et al.*, 2002, 2004), which corresponds to our findings. Landoni and Lees (1995b) observed 31% of inversion from R(–) to S(+) KETO, combined with a longer $t_{1/2el}$ for S(+) KETO. Igarza *et al.* (2002, 2004) reported a higher percentage of inversion in newborn calves (51%), as well as significantly different values for AUC and Cl between the enantiomers. Nevertheless, enantioselectivity in more than one PK process may be compensatory and in that way result

in similar AUC values for individual enantiomers after the administration of the drug racemate (Evans, 1992). In this respect, further research could elucidate the processes contributing to the observed differences, including possible age- or diet-related factors.

In conclusion, plasma concentrations of S(+) KETO predominated over R(–) KETO subsequent to the IM administration of racemic KETO in calves. In contrast with the previously reported PK observed after IV administration of the drug racemate, the results of the present study demonstrate distinct enantioselective differences between the PK properties of R(–) and S(+) KETO. This emphasizes the importance of enantioselective PK studies in comparison to total drug analyses, with respect to the understanding of pharmacological and toxicological consequences, as well as for the prediction of potential drug interactions and influences of the inflammatory state.

Acknowledgements

The authors would like to thank the Master student in Pharmaceutical Sciences, J. De Waele, for her excellent assistance in this study.

CHAPTER 3

In vivo studies of the immunomodulatory properties of veterinary drugs with respect to the acute-phase response

CHAPTER 3.1

Study of the immunomodulatory properties of gamithromycin and dexamethasone in lipopolysaccharide-challenged calves

Adapted from

Plessers, E., Watteyn, A., Wyns, H., Pardon, B., De Backer, P., Croubels, S., 2015. Immunomodulatory properties of gamithromycin and dexamethasone in lipopolysaccharide-challenged calves with emphasis on the acute-phase response.

(In preparation)

Abstract

The aim of this study was to define the *in vivo* immunomodulatory properties of the macrolide antibiotic gamithromycin (GAM) in calves, with respect to the acute-phase response. Additionally, the corticosteroid dexamethasone (DEX) was included as a positive control immunomodulatory drug. Both drugs, as well as their combination, were studied in a previously developed inflammation model, which was initiated by an IV LPS challenge (0.5 µg/kg body weight). The results were compared to those obtained previously in calves that received no pharmacological treatment (LPS-group; n = 9). In the present experiment, eighteen 4-week-old male Holstein Friesian calves were treated with GAM (n = 6), DEX (n = 6) or their combination (n = 6) 1 h prior to LPS administration. Blood collection and clinical scoring were performed at regular time points until 72 h p.c. Plasma concentrations of selected cytokines (TNF-α and IL-6) and acute-phase proteins (SAA and Hp) were subsequently determined. GAM did not have any effect on the LPS-induced clinical signs (dyspnea, fever, anorexia and depression), nor on the studied inflammatory mediators. In the DEX and combination group, the occurrence of dyspnea and fever was not prominently influenced, although the calves recovered significantly faster from the challenge. Moreover, DEX significantly inhibited the levels of TNF-α and IL-6, suggesting a key role for these cytokines in sickness behaviour. In conclusion, unlike DEX, GAM did not directly reduce cytokine release in an LPS inflammation model in calves.

1. Introduction

Acute inflammatory diseases in cattle, like bovine respiratory disease and mastitis, can evolve to dysregulated or excessive immune reactions, consequently impairing animal welfare (Bradley, 2002; Snowder *et al.*, 2007; Peri *et al.*, 2010). In this respect, the first reports suggesting beneficial effects of certain antimicrobial and (N)SAIDs on the innate immune system have led to high prospects of the research field of immunomodulation, particularly in regard to the treatment of endotoxemic or septicemic patients (Labro, 2000; Christaki *et al.*, 2011). Also the need for bovine inflammation models in the absence of active bacteria has been put forward repeatedly in view of the study of the immunomodulatory properties of antibiotics and anti-inflammatory drugs in cattle (Bednarek *et al.*, 2003; Buret, 2010). In this respect, an LPS-induced acute-phase response in calves can serve as a model to investigate the influence of drugs on the release of pro-inflammatory cytokines and acute-phase proteins, as well as on the clinical signs, including dyspnea, fever, anorexia and depression (Semrad, 1993a; Ohtsuka *et al.*, 1997a; EMA, 2001; Plessers *et al.*, 2015b). Moreover, this information could contribute to the development of improved treatment strategies, with emphasis on animal welfare (Zarogoulidis *et al.*, 2012).

Macrolide antibiotics have been demonstrated to possess immunomodulatory properties, independently of their antimicrobial actions (Labro, 2000; Giamarellos-Bourboulis, 2008; Kanoh and Rubin, 2010). In this respect, modulation of the release of pro-inflammatory cytokines and alteration of neutrophil functions are frequently reported effects. In cattle, both *in vitro* and *in vivo* studies have confirmed several immunomodulatory actions of tilmicosin and tulathromycin, including the induction of apoptosis in pulmonary neutrophils and the subsequent reduction of interleukin (IL)-8 and leukotriene B_4 synthesis (Buret, 2010; Fischer *et al.*, 2013). For the relatively recent azalide GAM (Fig. 3.1.1), on the other hand, data regarding its immunomodulatory behaviour are lacking (Huang *et al.*, 2010).

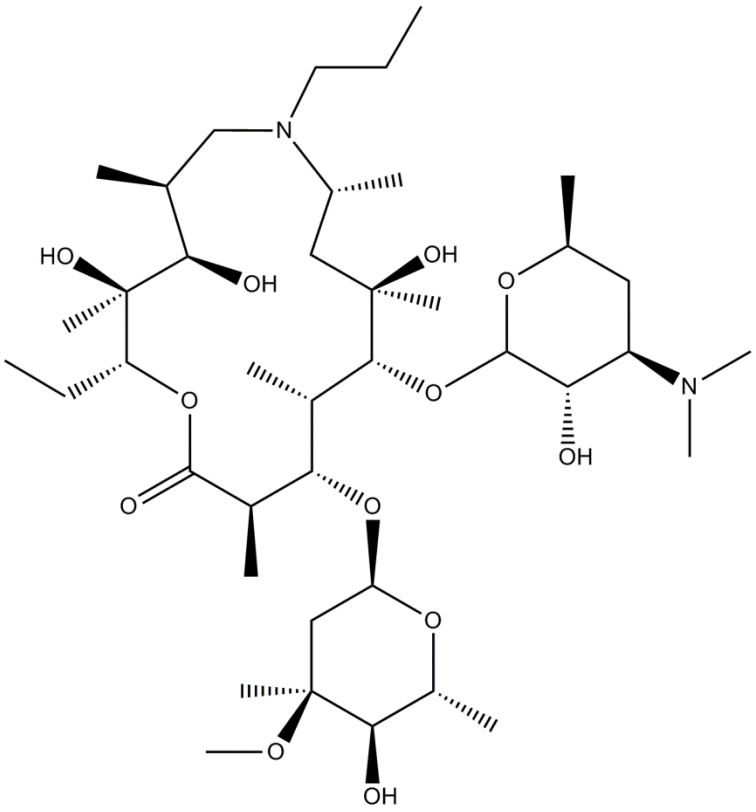

Figure 3.1.1. Chemical structure of gamithromycin (GAM)

Despite the immunosuppressive effect of corticosteroids, these drugs are frequently used in the treatment of acute inflammatory processes in cattle, based on their good short-term effects (Lekeux and Van de Weerdt, 1997). Accordingly, corticosteroids inhibit various components of the acute-phase response, including the onset of fever and the release of pro-inflammatory cytokines (Coelho *et al.*, 1995; Brattsand and Linden, 1996). In calves, Ohtsuka *et al.* (1997a) indeed demonstrated an inhibitory effect of DEX (Fig. 3.1.2) on LPS-induced TNF-α serum levels. From this point of view, DEX can be referred to as a positive control immunomodulatory drug.

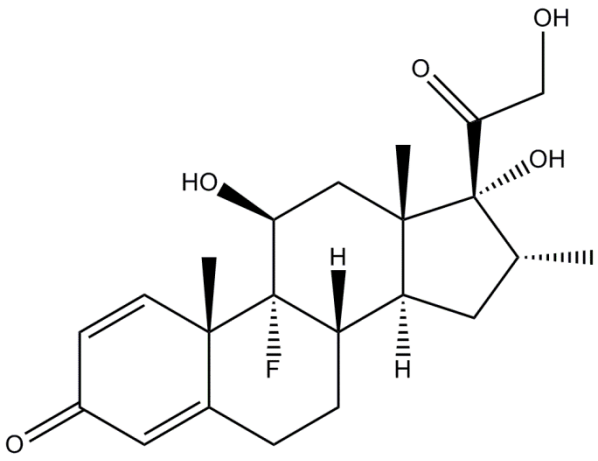

Figure 3.1.2. Chemical structure of dexamethasone (DEX)

The aim of this research was to study the immunomodulatory properties of GAM and DEX, as well as the combination of both drugs, in our previously developed *in vivo* LPS inflammation model in calves (Plessers *et al.*, 2015b). More specifically, emphasis was placed on the pro-inflammatory cytokines TNF-α and IL-6, the acute-phase proteins SAA and Hp, and the clinical signs following endotoxin challenge.

2. Materials and methods

2.1. Animal experiment

In order to evaluate the effects of the different pharmacological treatments, the results of the present study were compared to those obtained in a previous experiment, which was performed in a similar time frame (Plessers *et al.*, 2015b). The aim of the former study was to develop a reproducible inflammation model in 4-week-old calves, using nine animals, which were not treated pharmacologically prior to the LPS challenge (LPS group; n = 9). Furthermore, the effects of the challenge on the release of pro-inflammatory cytokines

(TNF-α and IL-6), acute-phase proteins (SAA and Hp), fever development and sickness behaviour were extensively described in this paper.

With respect to the present study, eighteen healthy male Holstein Friesian calves, with a mean age of 21.3 ± 5.8 days, were obtained from local farms. Upon arrival at the Faculty of Veterinary Medicine, the calves were housed and treated following a similar protocol as described in Plessers *et al.* (2015b). Briefly, the animals were housed in individual pens on straw with ad libitum access to hay and fresh water. The calves were fed milk replacer three times a day, receiving a total of 5 L daily. After the morning feeding, 50 g of starter mix was given to the calves. In order to evaluate the animals' clinical condition and to habituate the animals to human presence and contact, as well as to experimental manipulations, a one-week acclimatisation period was set. The day before the start of the experiment, the calves were weighed (56.8 ± 6.1 kg) after which a 14 G indwelling catheter (Cavafix, B. Braun, Diegem, Belgium) was placed aseptically in the right jugular vein, in order to facilitate serial blood collection. A recovery period of at least 12 h was respected. After this period, the 4-week-old calves were randomly divided into three groups: a GAM group (n = 6), a DEX group (n = 6) and a GAM-DEX group (n = 6). The clinical condition at 0 h was evaluated by determination of the rectal body temperature and visual inspection of the faeces. A rectal body temperature ≥ 39.5 °C was handled as an exclusion criterion at this time.

Reference venous blood samples (0 h) for cytokines and acute-phase proteins were collected from the catheter and transferred into EDTA-containing tubes. According to the group, the calves were subsequently treated with either 6 mg/kg BW GAM (Zactran®, Merial, Diegem, Belgium) subcutaneously (SC) in the neck region, 0.3 mg/kg BW DEX (Dexa® 0.2%, Kela Laboratoria, Hoogstraten, Belgium) IM in the neck region (cervical ventral serratus muscle), or the combination of both drugs (6 mg/kg BW GAM SC and 0.3 mg/kg BW DEX IM). One hour after drug administration, all calves were IV challenged with 0.5 µg/kg BW ultrapure LPS (500 units/kg BW, *E. coli* serotype O111:B4, LPS-EB Ultrapure, InvivoGen, Toulouse, France) via the catheter. Blood samples for cytokines and acute-phase proteins analyses were collected at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 18, 24, 28, 32, 48, 54 and 72 h p.c. At all mentioned sampling points, rectal body temperature (RT), respiratory rate (RR) and heart rate (HR) were recorded as well. Additionally, animals were clinically scored during

the first 9 h of the experiment, including the evaluation of the presence of dyspnea, coughing, breathing sounds, mental state, position and appetite. Based on these observations, the appearance of the three behavioural phases (respiratory, depression and recovery phase) following LPS administration was recorded (Plessers *et al.*, 2015b). If clinical signs of systemic shock would occur during the experiment, the respective calf would be humanely euthanased, and subsequently submitted for post-mortem examination. The investigators were not blinded to the treatment groups.

All blood samples were centrifuged at 1,000 x g for 15 min, after which plasma was harvested and stored in aliquots at ≤ -70 °C until analysis, with a maximum of 6 months.

All procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC 2011/057).

2.2. Sample analyses for cytokines and acute-phase proteins

Plasma samples were analysed in duplicate for cytokines and acute-phase proteins using commercially available ELISAs. As their suitability for plasma samples was not guaranteed by the manufacturer, the assays for cytokines (Bovine TNF-α DuoSet, R&D Systems Europe, Abingdon, UK; and Bovine IL-6 Screening Set, Thermo Fisher Scientific, Rockford, IL, USA) were validated prior to use (Plessers *et al.*, 2015b). The SAA (Phase SAA Assay, Tridelta Development Ltd., Maynooth, Ireland) and Hp (Haptoglobin Bovine ELISA, Alpco, Salem, NH, USA) assays were performed according to the manufacturer's protocol.

2.3. Statistical analysis

The results are presented as means (± SD or + SD). The effect of the LPS challenge within the different groups was assessed using repeated measures ANOVA. Additionally, area under the curve ($AUC_{0\rightarrow t}$) values for the time course of RT ($AUC_{0\rightarrow 24h}$), RR ($AUC_{0\rightarrow 6h}$), HR ($AUC_{0\rightarrow 24h}$) as well as TNF-α ($AUC_{0\rightarrow 6h}$), IL-6 ($AUC_{0\rightarrow 8h}$), SAA ($AUC_{0\rightarrow 72h}$) and Hp ($AUC_{0\rightarrow 72h}$) plasma concentrations were calculated, and subsequently analysed by single factor ANOVA

(Altan *et al.*, 2010). Pairwise comparisons of the mean AUC values of the different groups were performed using the Bonferroni test. For those parameters that were not normally distributed, the Kruskal-Wallis ANOVA by ranks was applied. Parameters that were only recorded once (including the occurrence of coughing, abnormal breathing sounds, open mouth breathing and the time of full recovery) were analysed using single factor ANOVA with Bonferroni-corrected pairwise comparisons. Differences were considered significant at $P < 0.05$. All analyses were performed in SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Clinical signs

Mean pre-LPS-challenge values for rectal body temperature, respiratory rate and heart rate among the different groups were 38.8 ± 0.2 °C, 35 ± 3 bpm and 75 ± 14 bpm, respectively. As can be observed from Table 3.1.1, Fig. 3.1.3 and Fig. 3.1.4, the effect of LPS was present in all groups, since these parameters altered significantly in time within each of the groups following LPS challenge ($P < 0.05$). Conversely, no significant differences were observed between the groups (Table 3.1.2). Normalization of the clinical signs was generally established within 24 h p.c. Two different visual trends regarding the course of the rectal body temperature were present in the LPS group, i.e. an early (n = 5) and delayed (n = 4) responding group, with a peak in rectal body temperature ≤ 5 h p.c. and ≥ 6 h p.c., respectively (Fig. 3.1.3). The DEX and GAM-DEX group showed a rectal body temperature course similar to the early responding LPS group, while the GAM group rather corresponded to the delayed responding LPS group. The respiratory rate raised sharply in all groups after LPS administration, reaching a peak at 0.5 h p.c., followed by a rather fast decline in the next hour (Fig. 3.1.4A). Regarding the heart rate, a consistent increase was observed within each of the groups (Fig. 3.1.4B), although the time of maximal heart rate differed among the groups.

Table 3.1.1. Mean (± SD) rectal body temperature (°C) after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 9) or following a pre-treatment with gamithromycin (GAM; n = 6), dexamethasone (DEX; n = 6) or the combination of both drugs (GAM-DEX; n = 6). The additional subdivision of the LPS group in an early (n = 5) and a delayed responding group (n = 4) is related to the time of maximal rectal body temperature (≤ 5 h or ≥ 6 h p.c.).

Time p.c. (h)	0	0.5	1	1.5	2	2.5	3	3.5	4	5	6	8	12	18	24
LPS Total	38.7 (0.4)	38.7 (0.3)	38.9 (0.2)	38.9 (0.3)	38.9 (0.3)	38.9 (0.4)	39.0 (0.5)	39.2 (0.6)	39.4 (0.5)	39.4 (0.2)	39.3 (0.5)	39.2 (0.5)	39.2 (0.6)	38.9 (0.4)	38.8 (0.2)
LPS Early	38.5 (0.4)	38.6 (0.2)	38.9 (0.2)	38.9 (0.2)	38.9 (0.2)	39.1 (0.2)	39.3 (0.3)	39.7 (0.4)	39.8 (0.2)	39.5 (0.1)	39.0 (0.5)	38.9 (0.5)	38.9 (0.5)	38.7 (0.3)	38.7 (0.3)
LPS Delayed	39.0 (0.3)	38.9 (0.4)	39.0 (0.2)	38.9 (0.4)	38.9 (0.5)	38.7 (0.5)	38.7 (0.4)	38.7 (0.4)	38.9 (0.4)	39.3 (0.3)	39.6 (0.1)	39.6 (0.2)	39.6 (0.3)	39.2 (0.2)	38.9 (0.1)
GAM	38.8 (0.2)	39.0 (0.3)	38.8 (0.3)	38.7 (0.3)	38.8 (0.5)	38.7 (0.5)	38.8 (0.7)	39.0 (0.7)	39.0 (0.8)	39.1 (0.7)	39.2 (0.5)	39.2 (0.3)	39.6 (0.6)	39.4 (0.5)	39.0 (0.3)
DEX	38.8 (0.2)	39.0 (0.2)	39.0 (0.4)	39.1 (0.3)	39.3 (0.4)	39.6 (0.6)	39.8 (0.6)	39.9 (0.4)	39.9 (0.4)	39.4 (0.2)	39.1 (0.2)	38.9 (0.2)	39.0 (0.2)	38.9 (0.1)	38.6 (0.1)
GAM-DEX	38.7 (0.1)	38.9 (0.3)	39.1 (0.3)	39.1 (0.3)	39.3 (0.4)	39.6 (0.4)	39.8 (0.4)	39.8 (0.4)	39.7 (0.4)	39.1 (0.2)	39.0 (0.3)	38.9 (0.4)	39.0 (0.4)	38.8 (0.3)	38.7 (0.3)

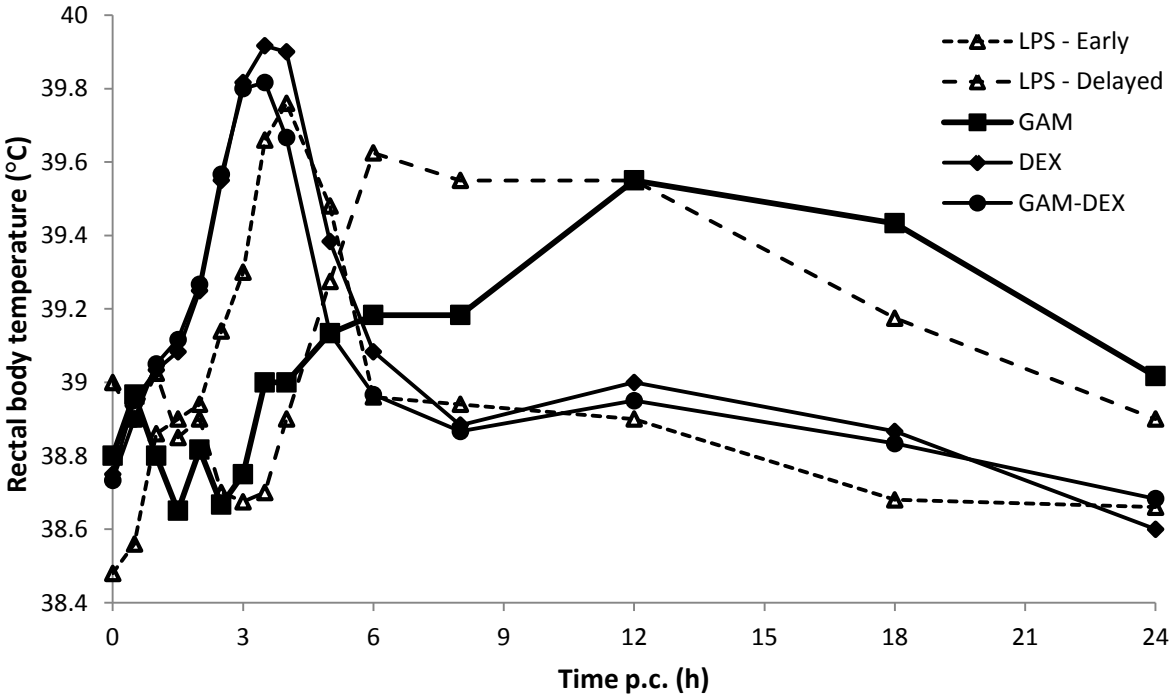

Figure 3.1.3. Time course of the mean rectal body temperature in 4-week-old calves after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS – Early responding group (n = 5) and LPS – Delayed responding group (n = 4)) or following pre-treatment with gamithromycin (GAM; n = 6), dexamethasone (DEX; n = 6) or the combination of both drugs (GAM-DEX; n = 6). For clarity of presentation, SDs are not included in this figure. However, Table 3.1.1 gives an overview of the full data set.

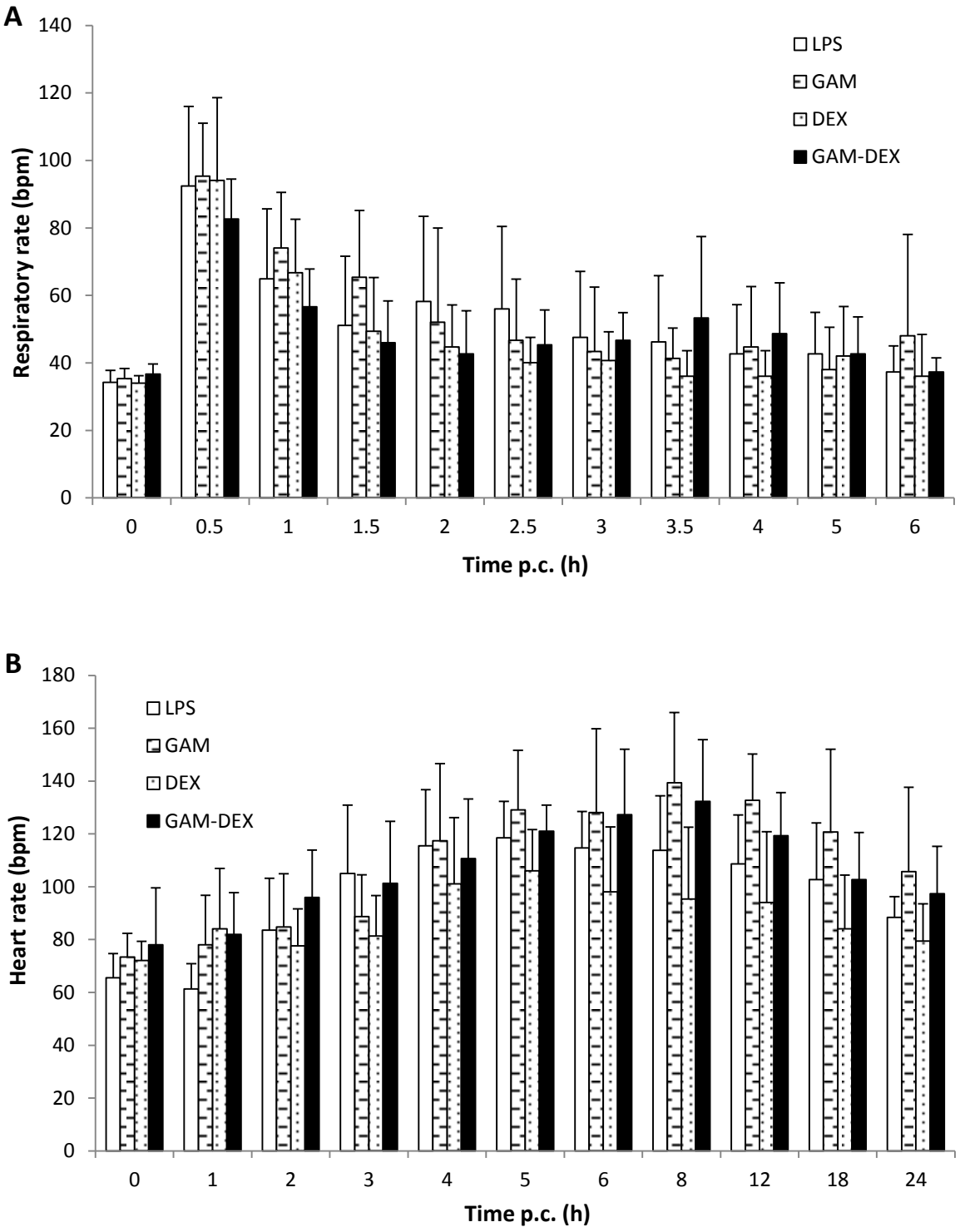

Figure 3.1.4. Mean (+SD) respiratory rate (A) and heart rate (B) in 4-week-old calves after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 9) or following pre-treatment with gamithromycin (GAM; n = 6), dexamethasone (DEX; n = 6) or the combination of both drugs (GAM-DEX; n = 6).

Table 3.1.2. Mean (± SD) area under the curve ($AUC_{0\rightarrow t}$) values for the time course of the rectal body temperature (RT), the respiratory rate (RR), the heart rate (HR), and TNF-α, IL-6, SAA and Hp plasma concentrations following an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 9) or following a pre-treatment with gamithromycin (GAM; n = 6), dexamethasone (DEX; n = 6) or the combination of both drugs (GAM-DEX; n = 6). Within a parameter, groups sharing a superscript letter do not differ from one another at the 5% global significance level.

	RT $\mathbf{AUC_{0\rightarrow 24h}}$ **(°C x h)**	**RR** $\mathbf{AUC_{0\rightarrow 6h}}$ **(bpm x h)**	**HR** $\mathbf{AUC_{0\rightarrow 24h}}$ **(bpm x h)**	**TNF-α** $\mathbf{AUC_{0\rightarrow 6h}}$ **(ng/mL x h)**	**IL-6** $\mathbf{AUC_{0\rightarrow 8h}}$ **(ng/mL x h)**	**SAA** $\mathbf{AUC_{0\rightarrow 72h}}$ **(mg/mL x h)**	**Hp** $\mathbf{AUC_{0\rightarrow 72h}}$ **(mg/mL x h)**
LPS	937[a] (7)	310[a] (83)	2448[a] (332)	25.9[a] (4.3)	60.1[a] (32.1)	12.6[a] (4.2)	5.3[a] (3.6)
GAM	942[a] (6)	313[a] (86)	2853[a] (503)	33.5[a] (14.4)	79.6[ab] (64.5)	12.8[a] (4.9)	11.3[a] (10.8)
DEX	936[a] (2)	281[a] (62)	2125[a] (423)	7.8[b] (5.4)	17.8[bc] (8.7)	10.3[a] (2.2)	21.1[a] (22.8)
GAM-DEX	935[a] (7)	294[a] (50)	2647[a] (369)	7.1[b] (6.6)	13.0[c] (9.0)	9.7[a] (2.7)	24.3[a] (18.9)

The LPS and GAM group demonstrated a rather similar course of the three successive behavioural phases, even though the intensity and duration of each phase varied among the individual animals (Fig. 3.1.5). None of the calves in these two groups showed any interest in starter mix, hay or water before the end of the recovery phase was reached. The calves in the DEX and GAM-DEX group, on the other hand, presented a number of remarkable deviations, including the absence of the depression phase and a faster recovery from the challenge.

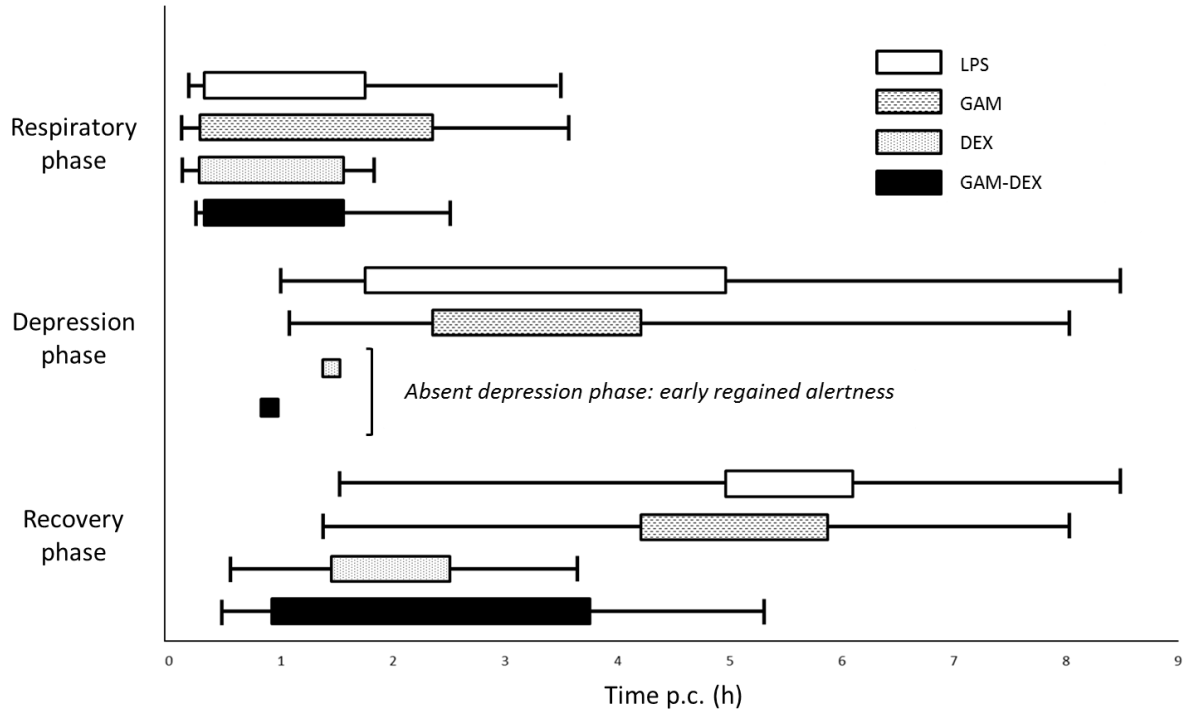

Figure 3.1.5. Time course of the three behavioural phases (respiratory, depression and recovery phase) in 4-week-old calves after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 9) or following pre-treatment with gamithromycin (GAM; n = 6), dexamethasone (DEX; n = 6) or the combination of both drugs (GAM-DEX; n = 6). This figure is based on scoring of the animals' clinical condition during the first nine hours of the experiment. In general, the boxes represent the mean duration of the different phases, whereas the vertical lines indicate the minimum and maximum points in time at which the respective phase starts and ends, within a certain calf. The squares in the DEX and GAM-DEX group indicate an exception as they represent the mean time at which alertness was regained. Hence, the depression phase was absent in these groups, as the calves became alert already during the respiratory phase.

During the respiratory phase, the calves were severely depressed, although respiratory symptoms predominated. Even though two and three calves of the DEX and GAM-DEX group, respectively, only showed moderate tachypnea (> 45 and ≤ 80 bpm), the occurrence of coughing (67-83%) and open-mouth breathing (17-33%) during the respiratory phase did not differ significantly among the four studied groups. Nevertheless, abnormal breathing sounds like stridor and stertor were absent in the DEX and GAM-DEX group, while in the remaining two groups, audible respiration occurred in approximately 50% of the animals. Most calves lay in sternal position, with their head and neck contacting the floor. Two calves in the LPS group and one calf in the GAM group already demonstrated lateral decubitus during this phase. Remarkably, alertness was regained prior to the recovery from respiratory distress in the DEX and GAM-DEX group (squares in Fig. 3.1.5).

For the calves in the LPS and GAM group, the depression phase was characterized by moderate depression that deteriorated while fever developed. During this phase, 67 and 33% of the calves in the LPS and GAM group, respectively, showed lateral decubitus over a certain period. Due to the early regained alertness of the calves in the DEX and GAM-DEX group (squares in Fig. 3.1.5), the depression phase was absent in these groups. Moreover, half of the DEX-treated calves already ate a small amount of starter mix prior to the fever peak (at approximately 2 h p.c.).

Since the depression phase was lacking following DEX administration, the calves in the DEX and GAM-DEX group recovered significantly faster than the calves in the LPS and GAM group. Full recovery, which was recognized by the moment at which the calf stands up, was observed in the LPS, GAM, DEX and GAM-DEX group at a mean time of 6.1, 5.9, 2.5 and 3.7 h p.c., respectively. Milk (1 L) was first presented 7 h p.c., and was refused by one calf in the LPS group and two calves in the GAM group at this time. At 13 h p.c., all calves easily consumed the supplied 2 L of milk and a handful (50 g) of starter mix.

3.2. Inflammatory mediators

Maximal levels of TNF-α, IL-6, SAA and Hp were attained at 1, 2.5-3.5, 24 and 18-28 h p.c., respectively, in all groups (Fig. 3.1.6 and 3.1.7). Nevertheless, cytokine concentrations were clearly reduced in the DEX and GAM-DEX group. For TNF-α, these declines were significant in comparison with both the LPS and the GAM group (Fig. 3.1.6A; Table 3.1.2). Also regarding IL-6, this overall lower trend following DEX pre-treatment persisted, although a significant difference could not be demonstrated between the GAM and the DEX group (Fig. 3.1.6B; Table 3.1.2). As described previously, IL-1β levels could not be determined due to the low concentrations and the required dilution factor for this assay (Plessers *et al.*, 2015b). With respect to SAA, DEX did not induce significant decreases, even though a slight inhibition of SAA-levels was perceived (Fig. 3.1.7A; Table 3.1.2). Regarding Hp, on the other hand, no stable trends could be observed among the DEX-treated groups, nor any significant differences between the groups (Fig. 3.1.7B; Table 3.1.2).

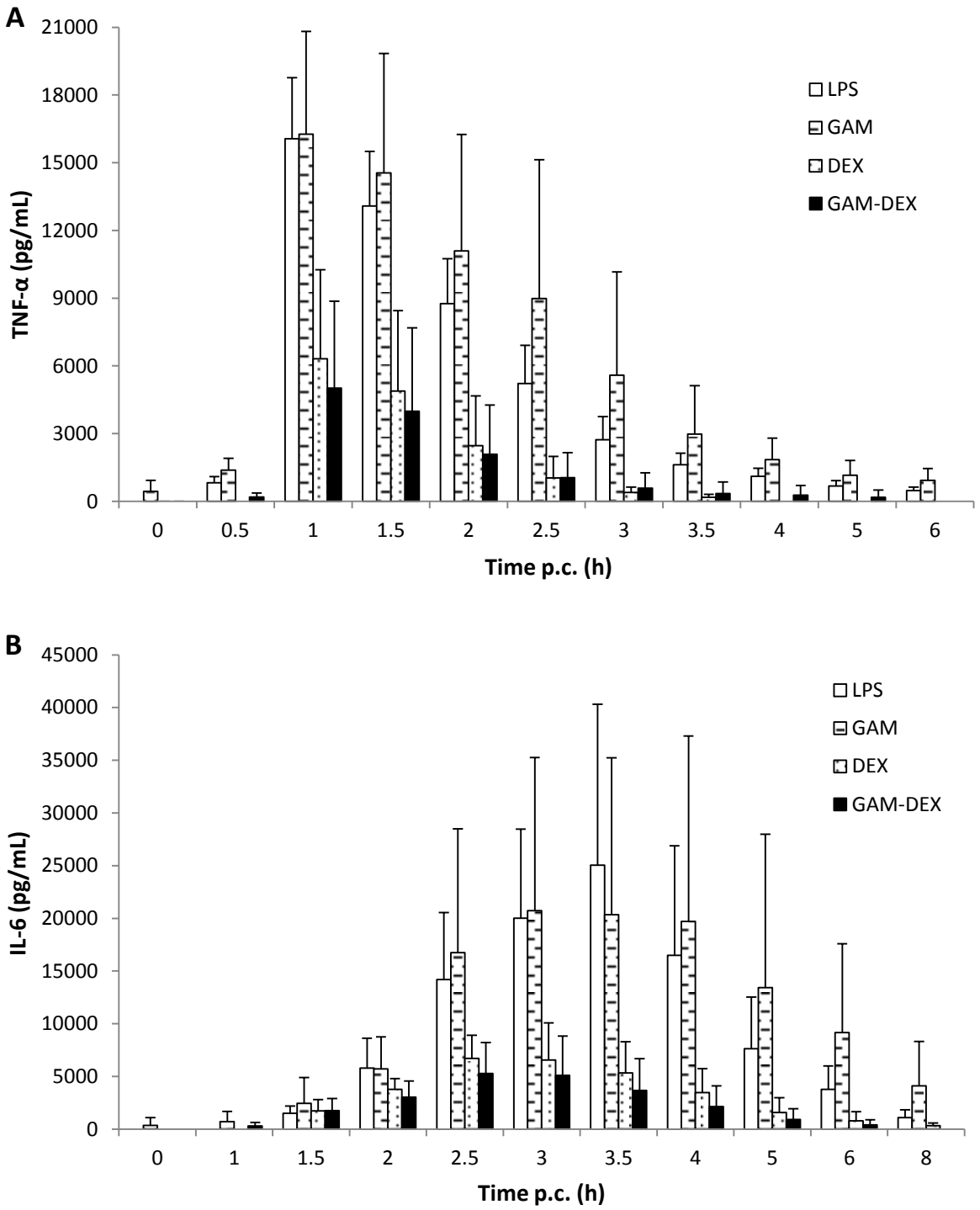

Figure 3.1.6. Mean (+SD) plasma concentrations of TNF-α (A) and IL-6 (B) in 4-week-old calves after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 9) or following pre-treatment with gamithromycin (GAM; n = 6), dexamethasone (DEX; n = 6) or the combination of both drugs (GAM-DEX; n = 6).

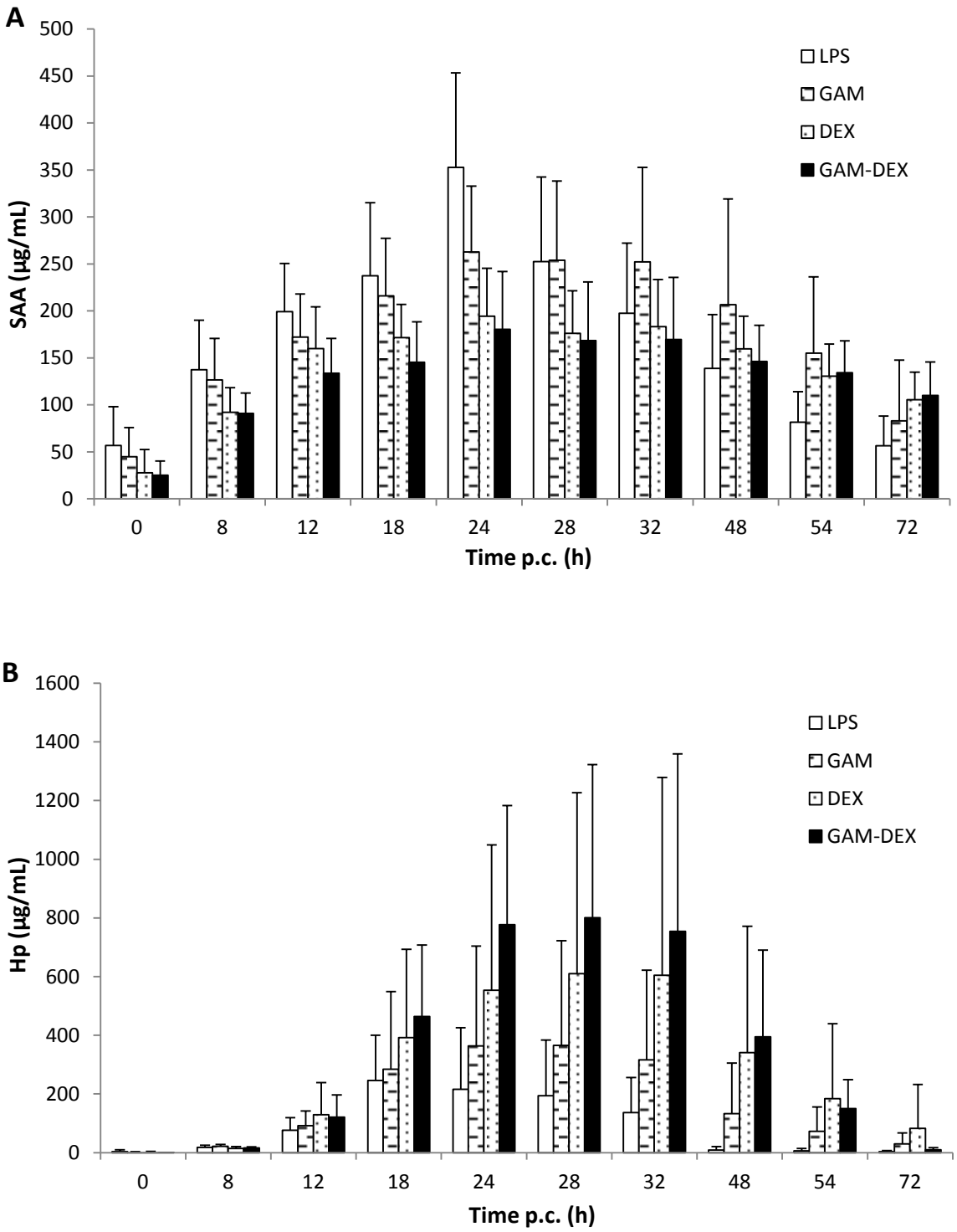

Figure 3.1.7. Mean (+SD) plasma concentrations of SAA (A) and Hp (B) in 4-week-old calves after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 9) or following pre-treatment with gamithromycin (GAM; n = 6), dexamethasone (DEX; n = 6) or the combination of both drugs (GAM-DEX; n = 6).

4. Discussion

In order to elicit a maximal effect of the drug on the innate immune response, all drugs were administered 1 h prior to LPS administration. This interval corresponds to the time of maximum plasma concentration of GAM (Huang *et al.*, 2010), as well as to the interval applied by Ohtsuka *et al.* (1997a) between DEX administration and the IV LPS bolus. Regarding GAM, the dose recommended by the manufacturer was applied, whereas for DEX, the dose used by Ohtsuka *et al.* (1997a) was administered, as the latter authors reported a clear influence of this dose on TNF-α release in calves.

The main objective of this study was to evaluate whether GAM, a second generation macrolide, indeed exerts immunomodulatory properties in calves. Drugs that exert both anti-bacterial as well as anti-inflammatory effects have been suggested to be most effective in treating bacteria-induced inflammatory diseases (Buret *et al.*, 2010). Nevertheless, in the present study, the administration of GAM had no influence on the LPS-induced clinical signs, nor on the studied inflammatory mediators. Similarly to the LPS group, depression deteriorated in the GAM group while the rectal body temperature increased. This group even demonstrated a rectal body temperature profile, more or less analogous to that of the delayed responding LPS group. A delayed rectal body temperature response was previously associated with a pre-shock effect in certain calves, consequently suggesting that GAM induced a more pronounced inflammatory reaction in comparison with the average of the LPS group (Lohuis *et al.*, 1988; Plessers *et al.*, 2015b). These findings do not correspond to previous reports on other macrolide antibiotics in cattle (Buret, 2010; Mazzilli and Zecconi, 2010; Fischer *et al.*, 2011). More specifically, tilmicosin, tylosin and tulathromcyin have all been confirmed to exert certain immunomodulatory actions in bovine *in vitro* and/or *in vivo* studies. Although the underlying mechanisms have yet to be fully characterized, the ability of tilmicosin and tulathromycin to induce neutrophil apoptosis is a recurrently reported effect (Chin *et al.*, 1998; Lee *et al.*, 2004; Fisher *et al.*, 2011). This is a highly important finding, as the production of pro-inflammatory mediators is inhibited in apoptotic cells. Nonetheless, the influence of macrolide antibiotics on the release of pro-inflammatory cytokines in cattle is a less explored research field. Besides the *in vitro* inhibition of transcription and secretion of the neutrophil attractant IL-8 by tulathromycin in LPS-

stimulated neutrophils and macrophages, only the downregulation of IL-1 and IL-6 by tylosin was reported in a *Staphylococcus aureus* challenged bovine cell line (Mazzilli and Zecconi, 2010; Fischer *et al.*, 2011, 2013). Also in other animal species, data from *in vivo* macrolide studies on the modulation of pro-inflammatory cytokine release are rather scarce, and principally limited to mice and rats (Ianaro *et al.*, 2000; Tkalcevic *et al.*, 2006; Leiva *et al.*, 2007). However, when interpreting the results of the GAM group of the present study, it should be kept in mind that a bacterial infection was lacking in the present study, and that this may also affect the clinical significance. From this point of view, the evaluation of GAM in a bacterial infection model could provide additional insights regarding its immunomodulatory properties.

In the present study, the potent long-acting corticosteroid DEX was selected as a positive control immunomodulatory drug. This choice was based on reports in cattle, confirming the capacity of DEX to inhibit the endotoxin-induced cardiopulmonary response, fever development and pro-inflammatory cytokine release (Olson and Brown, 1986; Lohuis *et al.*, 1989; Ohtsuka *et al.*, 1997a). The effects on the lung and rectal body temperature are generally attributed to the lipocortin-mediated inhibition of the formation of arachidonic acid metabolites, including TXA_2 and PGE_2 (Coelho *et al.*, 1995). The influence on the release of pro-inflammatory cytokines, on the other hand, is rather associated with repression of transcription factor NF-κB, which plays a key role in the activation of immunoregulatory genes in response to inflammatory stimuli (Auphan *et al.*, 1995; Smoak and Cidlowski, 2004). Nevertheless, the results from the present experiment did not correspond entirely to the previously mentioned studies, since respiratory distress and fever development following LPS challenge were not prominently influenced by pre-treatment with DEX. Still, DEX had a clear positive influence on recovery from the challenge, as the depression phase was absent in the DEX-treated groups. Also regarding TNF-α and IL-6, a marked inhibition was evoked by DEX, subsequently resulting in lower SAA levels. From this point of view, it can be put forward that depression is associated with cytokine release (particularly TNF-α). Previous research indeed established that peripherally produced pro-inflammatory cytokines induce sickness behaviour (Johnson, 2002; Dantzer, 2009). Regarding the discrepancy between the hypothesized and observed effects of DEX, it can be suggested that the inactivation of NF-κB occurs sooner than the inhibition of eicosanoid production, the latter being rather correlated

with fever development. Accordingly, binding of a glucocorticoid to its receptor results in a fast inhibition of NF-κB activity, while several hours of corticosteroid incubation are required for neutrophils to synthesize maximal lipocortin levels *in vitro* (Hirata, 1983; Smoak and Cidlowski, 2004). Overall, it can be concluded that the effects of DEX are not unambiguous. This is in accordance with findings in literature, as different studies on the efficacy of corticosteroids as (ancillary) therapy in the treatment of acute inflammatory processes have yielded controversial results (Christie *et al.*, 1977; Espinasse *et al.*, 1992; Semrad, 1993b; Sustronck *et al.*, 1997; Malazdrewich *et al.*, 2004b; Hewson *et al.*, 2011). In this respect, the determination of eicosanoid concentrations, including PGE_2 and TXA_2, might provide an improved insight into the different effects of DEX observed in the present study. However, as these analyses require a specific sampling protocol (i.e. addition of indomethacin in order to prevent *ex vivo* artefactual eicosanoid generation), these inflammatory mediators could not be determined for the present experiment.

5. Conclusions

This *in vivo* study in 4-week-old calves demonstrated that neither GAM nor DEX fully exerted their hypothesized immunodulatory actions following an IV endotoxin challenge. Nevertheless, pre-treatment with DEX resulted in a faster recovery, consequently improving animal welfare. The information obtained in the present study can therefore contribute to the development of improved treatment combinations and strategies, and can serve as a basis to compare the clinical efficacy of corticosteroids and NSAIDs.

Acknowledgements

The help of M. Devreese, J. Goossens, J. Lambrecht, A. Osselaere, E. Russo, V. Vandenbroucke and A. Van den Bussche during the animal experiment was greatly appreciated by the authors.

CHAPTER 3.2

Study of the immunomodulatory properties of gamithromycin and ketoprofen in lipopolysaccharide-challenged calves

Adapted from

Plessers, E., Wyns, H., Watteyn, A., Pardon, B., De Baere, S., De Backer, P., Croubels, S., 2015. Study of the immunomodulatory properties of gamithromycin and ketoprofen in a lipopolysaccharide inflammation model in calves.

(In preparation)

Abstract

Background: Macrolide antibiotics and NSAIDs have been reported to be modulators of the innate immune response, irrespectively of their antimicrobial and anti-inflammatory actions. The macrolide gamithromycin (GAM) and the NSAID ketoprofen (KETO) are frequently used drugs in bovine veterinary medicine.

Hypothesis: (1) Both GAM and KETO attenuate the acute-phase response following an intravenous LPS challenge in calves, resulting in a faster clinical recovery. (2) The combined administration of GAM and KETO is beneficial due to synergistic and/or additive effects.

Animals: Sixteen 4-week-old Holstein-Friesian calves.

Methods: Experimental study, based on a previously developed LPS inflammation model. The calves were randomized into 4 groups: LPS group (positive control) receiving no pharmacological treatment (n = 4), and GAM (n = 4), KETO (n = 4) and GAM-KETO (n = 4) group receiving the respective drugs 1 h prior to LPS challenge (0.5 µg/kg body weight). Clinical scoring was performed and plasma concentrations of the selected cytokines (TNF-α and IL-6), acute-phase protein (SAA) and PGE_2 were quantified.

Results: Pre-treatment with KETO had a remarkable effect in the LPS model, as depression, anorexia and fever were completely inhibited. PGE_2 synthesis was significantly reduced by KETO, whereas the effect on TNF-α, IL-6 and SAA was not straightforward. Pre-treatment with GAM had no effect compared to the LPS group and the combination treatment provided no synergistic or additive effects in this model, neither clinically nor regarding the studied inflammatory mediators.

Conclusion and clinical importance: KETO entirely inhibited fever development and depression, while GAM did not exert any effect in this model. The effect of KETO can be attributed to the complete reduction of PGE_2 levels. These results promote the concomitant use of an antimicrobial drug and a NSAID in the treatment of calf diseases associated with LPS, both to enhance clinical recovery and to improve animal welfare.

1. Introduction

Bacterial infections are highly common in bovine veterinary medicine, with LPS playing a key role in the pathogenesis of several of these clinical entities. Currently, the treatment of acute bacterial infections in cattle is mostly restricted to the administration of antimicrobial drugs (Edwards *et al.*, 2010; Pardon *et al.*, 2012a). However, both from the animal welfare and economic perspective, the concomitant use of an anti-inflammatory drug is advantageous as it decreases the severity of clinical symptoms and limits the negative consequences of inflammation (Francoz *et al.*, 2012). In this respect, the choice between a NSAID and a corticosteroid remains a controversial topic in cattle practice (Lekeux and Van de Weerdt, 1997). Although immunosuppression is a well-known side effect of corticosteroids, these drugs are still commonly preferred by practitioners for their potent anti-inflammatory properties (Sustronck *et al.*, 1997; Wagner and Apley, 2004; Hewson *et al.*, 2011; Sipka *et al.*, 2013). Indeed, a fast clinical effect on depression and anorexia is highly appreciated by farmers. Nevertheless, in our previous study in LPS challenged calves, DEX did not inhibit the onset of respiratory distress and fever (Plessers *et al.*, in preparation). A faster recovery of the calves, on the other hand, was observed as well as reduced levels of the pro-inflammatory cytokines TNF-α and IL-6 and the acute-phase protein SAA.

From this point of view, NSAIDs might exert a more pronounced influence on the LPS-induced acute-phase response in comparison with corticosteroids. Indeed, COX-inhibitors directly block the enzymatic production of prostanoids. As a result, the production of prostaglandins and thromboxanes ceases, consequently resolving fever and pulmonary effects (Lekeux and Van de Weerdt, 1997; Lees *et al.*, 2004). In addition to these COX-dependent actions, certain NSAIDs have been established to modulate the innate immune response, including the inhibition of NF-κB-related gene transcription (Tegeder *et al.*, 2001; Bryant *et al.*, 2003). In bovine veterinary medicine, KETO is a commonly used NSAID, exerting anti-inflammatory, antipyretic and analgesic effects (Lees *et al.*, 2004; Pardon *et al.*, 2012a).

Particularly with respect to the treatment of bovine respiratory disease, the combination of an antimicrobial agent with a NSAID (including carprofen, flunixin meglumine, KETO and meloxicam) has been demonstrated to be superior to the

administration of an antimicrobial alone (Lockwood *et al.*, 2003; Elitok and Elitok, 2004; Friton *et al.*, 2005). In this context, GAM is a recently developed azalide that has been approved for the treatment of bovine respiratory disease (Huang *et al.*, 2010). To date, studies comparing the efficacy of NSAIDs and corticosteroids in cattle are rather limited, whereas research regarding their possible synergistic and additive effects with GAM is lacking.

Therefore, the aim of this study was to investigate the immunomodulatory properties of KETO in a previously developed inflammation model in calves. The combined administration of GAM and KETO was included in the study to test for synergistic and/or additive effects. Besides the effect on TNF-α, IL-6, SAA and clinical signs, emphasis was placed on the impact on prostanoids, i.e. PGE_2.

2. Materials and methods

2.1. Animal experiment

Twenty-four healthy male Holstein Friesian calves, with a mean age of 21.1 ± 4.0 days, were obtained from local farms. Upon arrival at the Faculty of Veterinary Medicine, the calves were housed and treated following a similar protocol as described in Plessers *et al.* (2015b). Briefly, the animals were housed in individual pens on straw with ad libitum access to hay and fresh water. The calves were fed milk replacer three times a day, receiving a total of 5 L daily. After the morning feeding, 50 g of starter mix was given to the calves. In order to evaluate the calves' clinical condition, and to habituate the animals to human presence and contact, as well as to experimental manipulations, a one-week acclimatization period was set.

The calves were weighed the day before the start of the experiment (56.1 ± 6.9 kg), after which a 14 G indwelling catheter (Cavafix, B. Braun, Diegem, Belgium) was placed aseptically in the right jugular vein. A recovery period of at least 12 h was respected. After this period, the 4-week-old calves were randomly divided into four groups: an LPS group (n = 6), a GAM group (n = 6), a KETO group (n = 6) and a GAM-KETO group (n = 6). Negative

control animals were not included in the present study, as our previous research demonstrated that these animals showed no changes regarding clinical condition, nor with respect to cytokine and acute-phase protein concentrations (Plessers *et al.*, 2015b). The clinical condition at 0 h was evaluated by determination of the rectal body temperature and visual inspection of the faeces. A rectal body temperature ≥ 39.5 °C was handled as an exclusion criterion at this time.

Reference venous blood samples (0 h) for cytokines and SAA were drawn from the catheter and transferred into EDTA-containing tubes. Additionally, reference blood samples for PGE_2 determination were collected by adding 1 mL of blood into Eppendorf tubes previously coated with 10 µg indomethacin (Sigma Aldrich, Diegem, Belgium) and containing 10 IU of sodium heparine (Heparine LEO, Leo Pharma, Lier, Belgium). The indomethacin was added in order to prevent *ex vivo* artefactual eicosanoid generation (Pelligand *et al.*, 2012). Briefly, 100 µL of the stock solution of indomethacin (0.1 mg/mL in ethanol (VWR, Leuven, Belgium)) were added per tube, and evaporated to dryness under a gentle stream of nitrogen (N_2) at 35 °C. According to the group, the calves were subsequently either not treated (LPS group), treated with 6 mg/kg BW GAM (Zactran®, Merial, Diegem, Belgium) SC in the neck region, treated with 3 mg/kg BW KETO (Ketofen® 10%, Merial, Diegem, Belgium) IM in the neck region (cervical ventral serratus muscle), or treated with the combination of both drugs (6 mg/kg BW GAM SC and 3 mg/kg BW KETO IM). Following a one-hour interval, all calves were IV challenged with 0.5 µg/kg BW ultrapure LPS (500 units/kg BW, *E. coli* serotype O111:B4, LPS-EB Ultrapure, InvivoGen, Toulouse, France) via the catheter. Blood samples were collected at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 18, 24, 28, 32, 48, 54 and 72 h p.c. At all mentioned sampling points, rectal body temperature (RT), respiratory rate (RR) and heart rate (HR) were recorded. Additionally, animals were clinically scored during the first 9 h of the experiment, including the evaluation of the presence of dyspnea, coughing, breathing sounds, mental state, position and appetite. Based on these observations, the appearance of three behavioural phases (respiratory, depression and recovery phase) following LPS administration was recorded (Plessers *et al.*, 2015b). If systemic shock symptoms would occur, the respective calf would be humanely euthanized, and subsequently necropsied. The investigators were not blinded to the treatment groups.

All blood samples were mixed by gentle inversion and placed on ice until centrifugation at 1,000 × g for 15 min. Subsequently, plasma was harvested and stored in aliquots at ≤ -70 °C for future analysis, with a maximum of 10 months.

2.2. Sample analyses for TNF-α, IL-6 and SAA

All plasma samples were analysed in duplicate for cytokines (TNF-α and IL-6) and the acute phase protein (SAA) using commercially available ELISAs. As their suitability for plasma samples was not guaranteed by the manufacturer, the assays for cytokines (Bovine TNF-α DuoSet, R&D Systems Europe, Abingdon, UK; and Bovine IL-6 Screening Set, Thermo Fisher Scientific, Rockford, IL, USA) were validated prior to use (Plessers *et al.*, 2015b). The SAA assay (Phase SAA Assay, Tridelta Development Ltd., Maynooth, Ireland) was performed according to the manufacturer's protocol.

2.3. Sample analyses for PGE_2

PGE_2 is rapidly converted by the prostaglandin 15-dehydrogenase pathway to its inactive metabolite 13,14-dihydro-15-keto PGE_2, which in turn is subject to further metabolism to 13,14-dihydro-15-keto PGA_2 (PGE_2-*met*) (Fig. 3.2.1) (Hamberg and Samuelsson, 1971; Granström *et al.*, 1980). The concentration of PGE_2 and PGE_2-*met* in plasma was determined using an in-house developed and validated ultra-performance liquid chromatography-tandem mass spectrometry method (UPLC-MS/MS) (De Baere *et al.*, unpublished data). Standards of PGE_2 and PGE_2-*met*, as well as the internal standard, deuterated PGE_2 (PGE_2-d_4), were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). UPLC grade methanol, acetonitrile and water were purchased from Biosolve (Valkenswaard, The Netherlands). Stock solutions of 1 mg/mL of PGE_2, PGE_2-*met* and PGE_2-d_4 were prepared in UPLC methanol and stored at ≤ -70 °C. Mixed working solutions of PGE_2 and PGE_2-*met* of 0.1, 1.0 and 10 ng/mL; and an internal standard working solution of PGE_2-d_4 of 10 ng/mL were prepared in UPLC methanol and stored at ≤ -70 °C.

Briefly, the sample preparation for 100 µL of plasma was performed by protein precipitation using 525 µL of acetonitrile, followed by centrifugation (10 min, 7825 x *g*, 4 °C), evaporation of the organic phase under N_2 and analysis of a 10 µL-aliquot by UPLC-MS/MS.

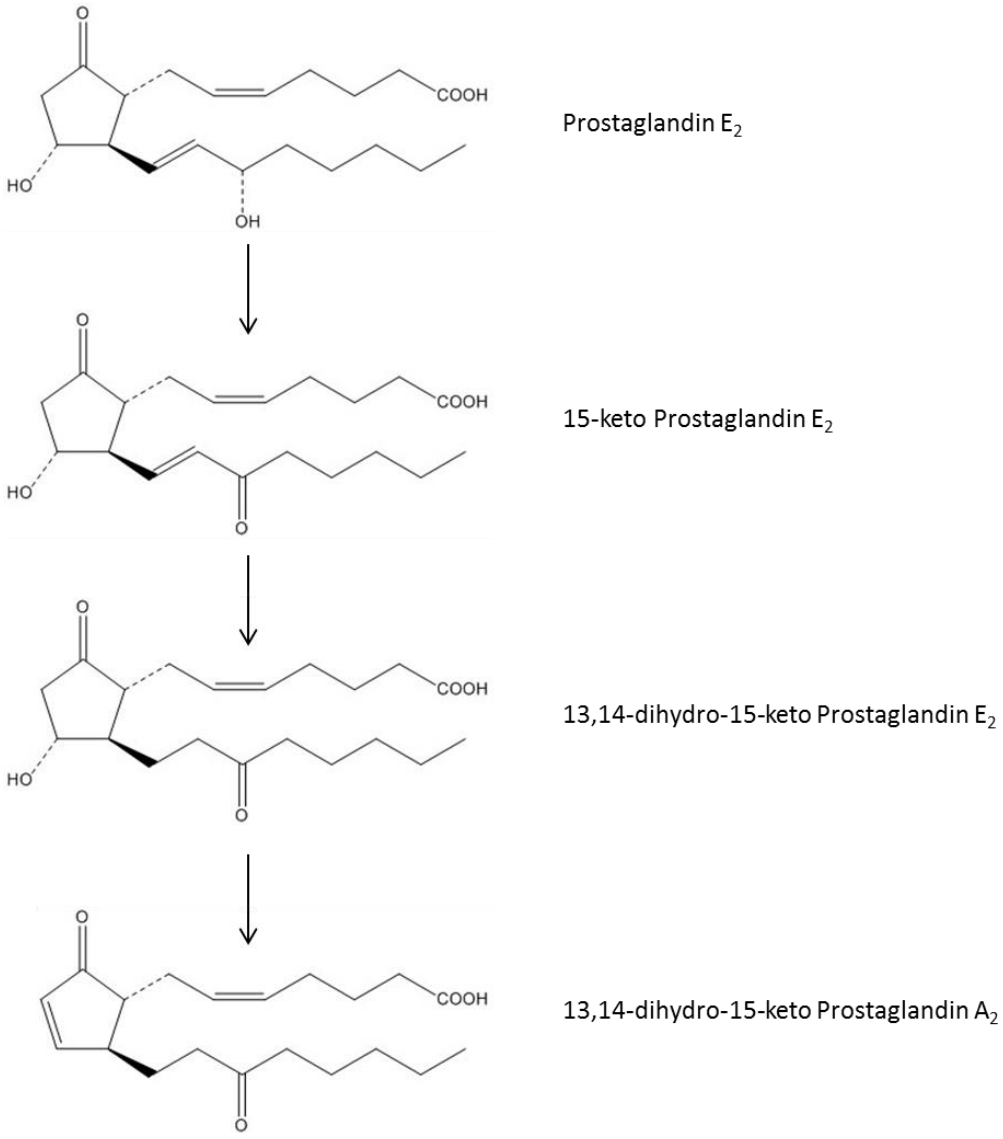

Fig. 3.2.1. Metabolism of PGE_2

The LC system consisted of an Acquity UPLC H-Class Quaternary Solvent Manager and Flow-Through-Needle Sample Manager with temperature controlled tray and column oven from Waters. Chromatographic separation was achieved on an Acquity UPLC BEH C18 column (50 mm x 2.1 mm i.d., dp: 1.7 µm) in combination with an Acquity BEH C18 Vanguard pre-column (Waters, Zellik, Belgium). The mobile phase A consisted of 0.01% formic acid in water, while the mobile phase B was 0.01% formic acid in acetonitrile. The UPLC column effluent was interfaced to a XEVO TQ-S MS/MS system, equipped with an electrospray ionization probe operating in the negative mode (all from Waters). A divert valve was used and the UPLC effluent was directed to the mass spectrometer from 2.0 to 4.9 min. MS/MS acquisition was performed in the multiple reaction monitoring (MRM) mode. The MRM transitions monitored as quantification and identification ion, respectively, were *m/z* = 351.22 > 271.26 and 351.22 > 315.21 for PGE_2; *m/z* = 355.16 > 319.28 and 355.16 > 275.27 for PGE_2-d4; *m/z* = 333.20 > 175.00 and 333.20 > 235.00 for PGE_2-*met*. Data for PGE_2-*met* were generated using an external standard method.

The method was validated by a set of parameters that were in compliance with the recommendations defined by EU standards (Commission Decision 2002/657/EC, VICH GL49, Heitzman, 1994) and by Knecht and Stork (1974). In this respect, linearity, within- and between-run accuracy and precision, limit of quantification (LOQ), limit of detection (LOD) and specificity were evaluated. Linear calibration curves (r > 0.99) were constructed in a concentration range of 50 to 5000 pg/mL. Within- and between-run accuracy and precision were evaluated by analysis of independently spiked samples (n = 6) at four concentration levels i.e. 50, 125, 500 and 5000 pg/mL, respectively. The results fell within the accepted ranges for accuracy and precision (2002/657/EC; VICH GL49). The LOQ was defined as the lowest concentration for which the method was validated with a within-run accuracy and precision that fell within the specified ranges and was established at 125 and 50 pg/mL for PGE_2 and PGE_2-*met*, respectively. The LOD was defined as the concentration corresponding with a signal-to-noise ratio of 3 and was found to be 16.7 and 6.8 pg/mL for PGE_2 and PGE_2-*met*, respectively. The specificity of the method was proven, since no peaks of endogenous interferences could be determined in blank samples.

2.4. Statistical analysis

The results are presented as means (± SD or + SD). The effect of the LPS challenge on the studied parameters within the different groups was assessed using repeated measures ANOVA. Additionally, area under the curve ($AUC_{0\rightarrow t}$) values for the time course of RT ($AUC_{0\rightarrow 24h}$), RR ($AUC_{0\rightarrow 6h}$), HR ($AUC_{0\rightarrow 24h}$) as well as TNF-α ($AUC_{0\rightarrow 6h}$), IL-6 ($AUC_{0\rightarrow 12h}$), SAA ($AUC_{0\rightarrow 72h}$) and PGE_2 ($AUC_{0\rightarrow 4h}$) plasma concentrations were calculated, and subsequently analysed by single factor ANOVA (Altan *et al.*, 2010). Pairwise comparisons of the mean AUC values of the different groups were performed using the Bonferroni test. For those parameters that were not normally distributed, an ln-transformation was executed. Parameters that were only recorded once in the LPS and GAM group, i.e. the duration of the behavioural phases, were analysed using an independent samples t-test. Differences were considered significant at $P < 0.05$. All analyses were performed using commercial statistic software (SPSS 22 software for Windows, IBM Corp., Armonk, NY, USA).

2.5. Ethical approval

All procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine and Faculty of Bioscience Engineering of Ghent University (EC 2012/189).

3. Results

The final number of calves in each of the groups was reduced from six to four. In the LPS group, two calves were excluded from the experiment as a rectal body temperature > 39.5 °C was measured at 0 h (40.4 and 40.7 °C, respectively). Two calves in the GAM group were euthanized at 3 and 4.75 h p.c., respectively, due to severe systemic shock symptoms. The subsequent necropsy and histological examination revealed acute diffuse alveolar damage, which was comparable to the lesions observed in a euthanized calf in our previous study (Plessers *et al.*, 2015b). Regarding the KETO and GAM-KETO group, two calves of each group

were excluded following cytokine analysis. More specifically, excessive cytokine release or an absent response were observed in these calves.

3.1. Clinical signs

Mean pre-LPS-challenge values for rectal body temperature, respiratory rate and heart rate among the different groups were 38.9 ± 0.2 °C, 32 ± 4 bpm and 80 ± 9 bpm, respectively. Figure 3.2.2, Figure 3.2.3, Table 3.2.1 and Table 3.2.2 demonstrate the unequivocal influence of pre-treatment with KETO on the clinical signs following LPS challenge. In this respect, fever development was completely inhibited in the KETO and GAM-KETO group. In the LPS and GAM group, on the other hand, a marked peak in rectal body temperature was observed 4 h p.c. The respiratory rate increased significantly in time within all groups following LPS administration (Fig. 3.2.3A), although the initial sharp rise, which was clearly induced in the LPS and GAM group, was absent in the KETO and GAM-KETO group. Nevertheless, a moderate increase in respiratory rate (> 40 and ≤ 80 bpm) was observed 1 h p.c. in 3 out of 4 animals in the latter two groups. As can be perceived in Figure 3.2.3B, the heart rate increased significantly in time within all groups as well, while no significant differences were observed between the groups.

As expected from the results for rectal body temperature and respiratory rate, the three successive behavioural phases following LPS challenge, were noticeably influenced by KETO pre-treatment (Fig. 3.2.4). More specifically, the common sequence which was present in the LPS and GAM group – the respiratory, depression and recovery phase – was abolished in the KETO and GAM-KETO group. Firstly, as demonstrated by the respiratory rate, no severe respiratory distress occurred in these groups. Instead, only moderate tachypnea was observed in 3 out of 4 calves. This effect was not initiated simultaneously with the start of the respiratory phase in the LPS and GAM group, yet showed a delay of approximately 30 minutes. Secondly, the depression phase, and consequently the recovery phase, were completely absent in the KETO-treated groups. The comparison of the duration of each of the three phases between the LPS and GAM group, on the other hand, revealed no statistical significant differences.

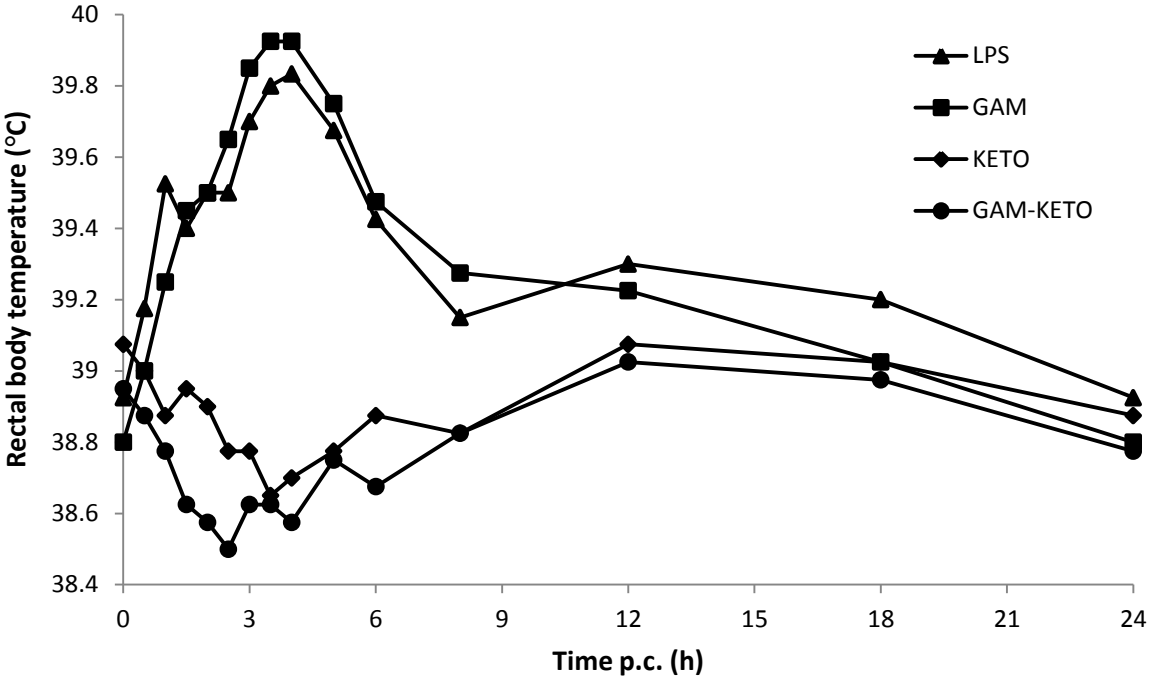

Figure 3.2.2. Time course of the mean rectal body temperature in 4-week-old calves after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 4) or following pre-treatment with gamithromycin (GAM; n = 4), ketoprofen (KETO; n = 4) or the combination of both drugs (GAM-KETO; n = 4). For clarity of presentation, SDs are not included in this figure. However, Table 3.2.1 gives an overview of the full data set.

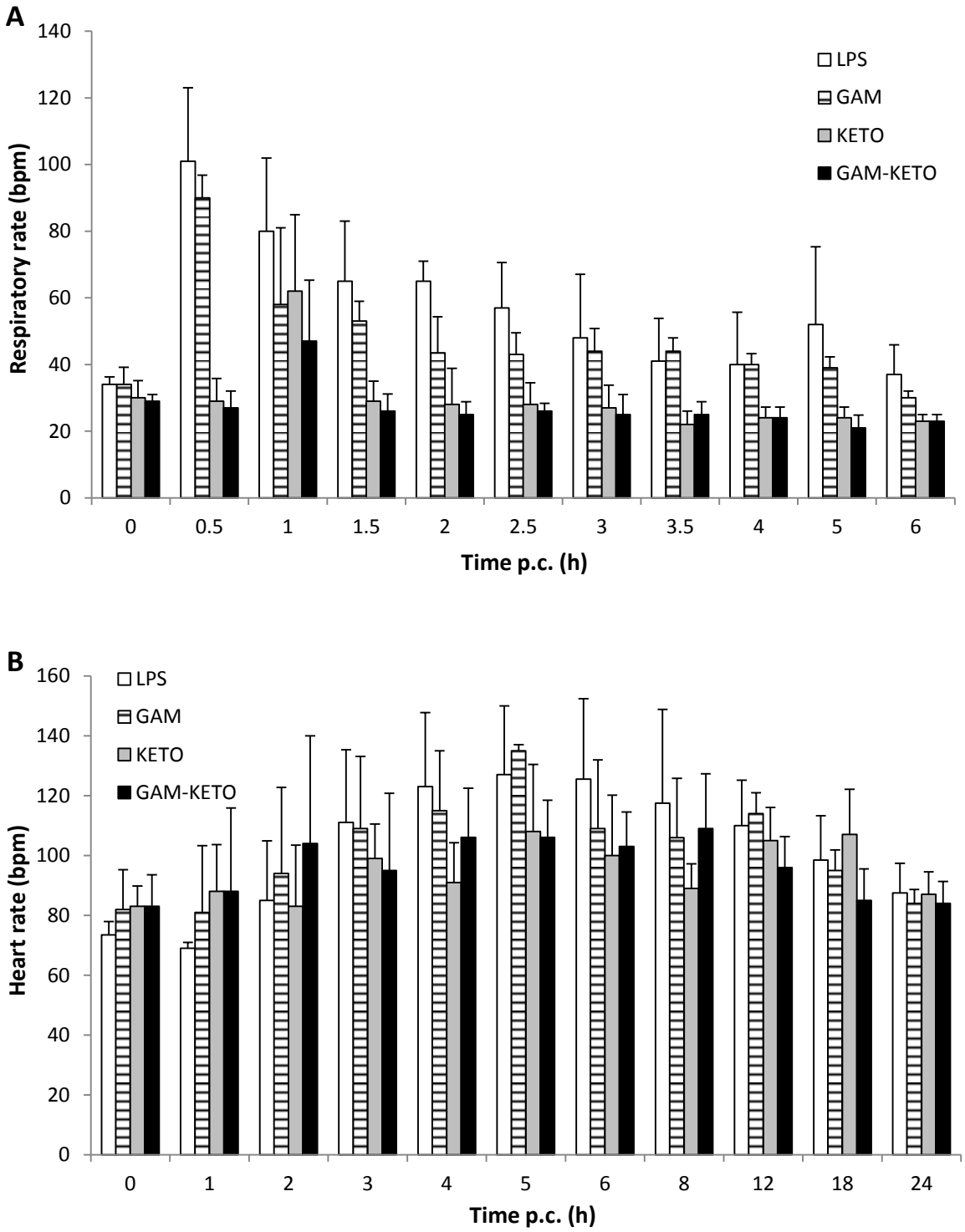

Figure 3.2.3. Mean (+SD) respiratory rate (A) and heart rate (B) in 4-week-old calves after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 4) or following pre-treatment with gamithromycin (GAM; n = 4), ketoprofen (KETO; n = 4) or the combination of both drugs (GAM-KETO; n = 4).

Table 3.2.1. Mean (± SD) rectal body temperature (°C) after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 4) or following a pre-treatment with gamithromycin (GAM; n = 4), ketoprofen (KETO; n = 4) or the combination of both drugs (GAM-KETO; n = 4).

Time p.c. (h)	0	0.5	1	1.5	2	2.5	3	3.5	4	5	6	8	12	18	24
LPS	38.9 (0.2)	39.2 (0.2)	39.5 (0.2)	39.4 (0.1)	39.5 (0.3)	39.5 (0.7)	39.7 (0.5)	39.8 (0.6)	39.8 (0.5)	39.7 (0.5)	39.4 (0.6)	39.2 (0.3)	39.3 (0.3)	39.2 (0.1)	38.9 (0.2)
GAM	38.8 (0.1)	39.0 (0.3)	39.3 (0.2)	39.5 (0.3)	39.5 (0.2)	39.7 (0.2)	39.9 (0.3)	39.9 (0.2)	39.9 (0.2)	39.8 (0.3)	39.5 (0.2)	39.3 (0.1)	39.2 (0.0)	39.0 (0.1)	38.8 (0.0)
KETO	39.1 (0.1)	39.0 (0.2)	38.9 (0.1)	39.0 (0.1)	38.9 (0.1)	38.8 (0.2)	38.8 (0.2)	38.7 (0.1)	38.7 (0.1)	38.8 (0.1)	38.9 (0.1)	38.8 (0.1)	39.1 (0.1)	39.0 (0.1)	38.9 (0.1)
GAM-KETO	39.0 (0.3)	38.9 (0.3)	38.8 (0.2)	38.6 (0.1)	38.6 (0.1)	38.5 (0.0)	38.6 (0.1)	38.6 (0.1)	38.6 (0.1)	38.8 (0.1)	38.7 (0.1)	38.8 (0.1)	39.0 (0.2)	39.0 (0.2)	38.8 (0.2)

Table 3.2.2. Mean (± SD) area under the curve ($AUC_{0\rightarrow t}$) values for the time course of the rectal body temperature (RT), the respiratory rate (RR), the heart rate (HR), and TNF-α, IL-6, SAA and PGE_2-*met* plasma concentrations following an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 4) or following pre-treatment with gamithromycin (GAM; n = 4), ketoprofen (KETO; n = 4) or the combination of both drugs (GAM-KETO; n = 4). Within a parameter, groups sharing a superscript letter do not differ from one another at the 5% global significance level. N/A: not applicable, as all values were below the LOQ.

	RT $AUC_{0\rightarrow 24h}$ **(°C x h)**	**RR** $AUC_{0\rightarrow 6h}$ **(bpm x h)**	**HR** $AUC_{0\rightarrow 24h}$ **(bpm x h)**	**TNF-α** $AUC_{0\rightarrow 6h}$ **(ng/mL x h)**	**IL-6** $AUC_{0\rightarrow 12h}$ **(ng/mL x h)**	**SAA** $AUC_{0\rightarrow 72h}$ **(mg/mL x h)**	**PGE_2-*met*** $AUC_{0\rightarrow 4h}$ **(pg/mL x h)**
LPS	943^{a} (3)	338^{a} (70)	2501^{a} (353)	17.2^{ab} (6.4)	108.8^{a} (74.8)	13.0^{a} (4.6)	512^{a} (345)
GAM	941^{a} (1)	280^{a} (78)	2448^{a} (186)	14.8^{ab} (15.0)	85.5^{a} (103.7)	12.7^{a} (5.1)	273^{a} (247)
KETO	934^{b} (1)	174^{b} (8)	2349^{a} (212)	5.2^{a} (4.1)	10.8^{a} (5.7)	5.2^{a} (1.9)	N/A
GAM-KETO	933^{b} (3)	158^{b} (23)	2262^{a} (222)	28.3^{b} (9.2)	83.6^{a} (44.0)	11.3^{a} (3.2)	N/A

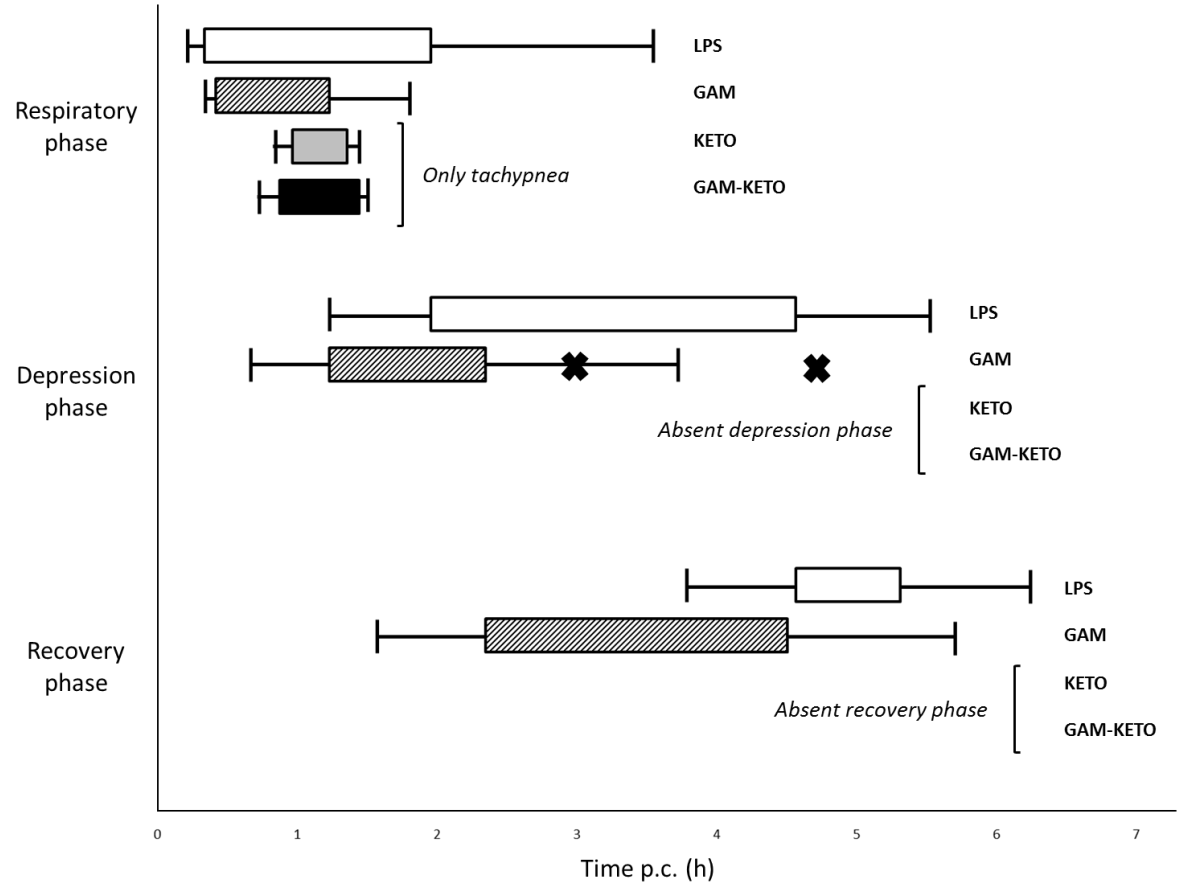

Figure 3.2.4. Time course of the three behavioural phases (respiratory, depression and recovery phase) in 4-week-old calves after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 4) or following pre-treatment with gamithromycin (GAM; n = 4), ketoprofen (KETO; n = 4) or the combination of both drugs (GAM-KETO; n = 4). This figure is based on scoring of the animals' clinical condition during the first nine hours of the experiment. In general, the boxes represent the mean duration of the different phases, whereas the vertical lines indicate the minimum and maximum points in time at which the respective phase starts and ends, within a certain calf. The x-marks represent the time of euthanasia of the two calves in the GAM group.

3.2. Inflammatory mediators

Maximal levels of TNF-α, IL-6 and SAA were attained in all groups at similar points in time as observed in our previous experiment (at 1, 2.5-3.5 and 24 h p.c., respectively) (Fig. 3.2.5 and 3.2.6) (Plessers *et al.*, in preparation). One calf of the KETO and one calf of the GAM-KETO group were excluded due to excessive cytokine concentrations following the LPS challenge (Fig. 3.2.5). The second calf in the KETO group was excluded as continuous high levels of TNF-α were detected during the experiment, while the cause of exclusion of the second calf in the GAM-KETO group was the lacking response to the challenge.

As can be concluded from Figure 3.2.5, quite opposite effects on the cytokine release were observed in the KETO and GAM-KETO group. For TNF-α, the administration of merely KETO decreased the plasma concentrations, whereas the combination of GAM and KETO resulted in higher TNF-α levels compared to the LPS and GAM group (Fig. 3.2.5A). In this respect, a significant difference was found between the KETO and GAM-KETO group (Table 3.2.2). Regarding IL-6, KETO on its own again reduced this cytokine's release, whereas no influence was observed in the GAM-KETO group (Fig. 3.2.5B; Table 3.2.2). As shown in Figure 3.2.6, this trend recurred with respect to SAA. Figure 3.2.7 depicts the profile of PGE_2-*met* following the endotoxin challenge. The metabolite was selected, as its LOQ was lower in comparison with PGE_2. In the LPS and GAM group, a marked increase of the levels of PGE_2-*met* was observed, as soon as 0.5 h p.c. The GAM group showed a tendency towards a faster normalization, although this was not significant (Table 3.2.2). The pre-treatment with KETO, on the other hand, resulted in a highly significant inhibition of PGE_2 production, as no quantifiable concentrations were measured during the experiment in the KETO and GAM-KETO group.

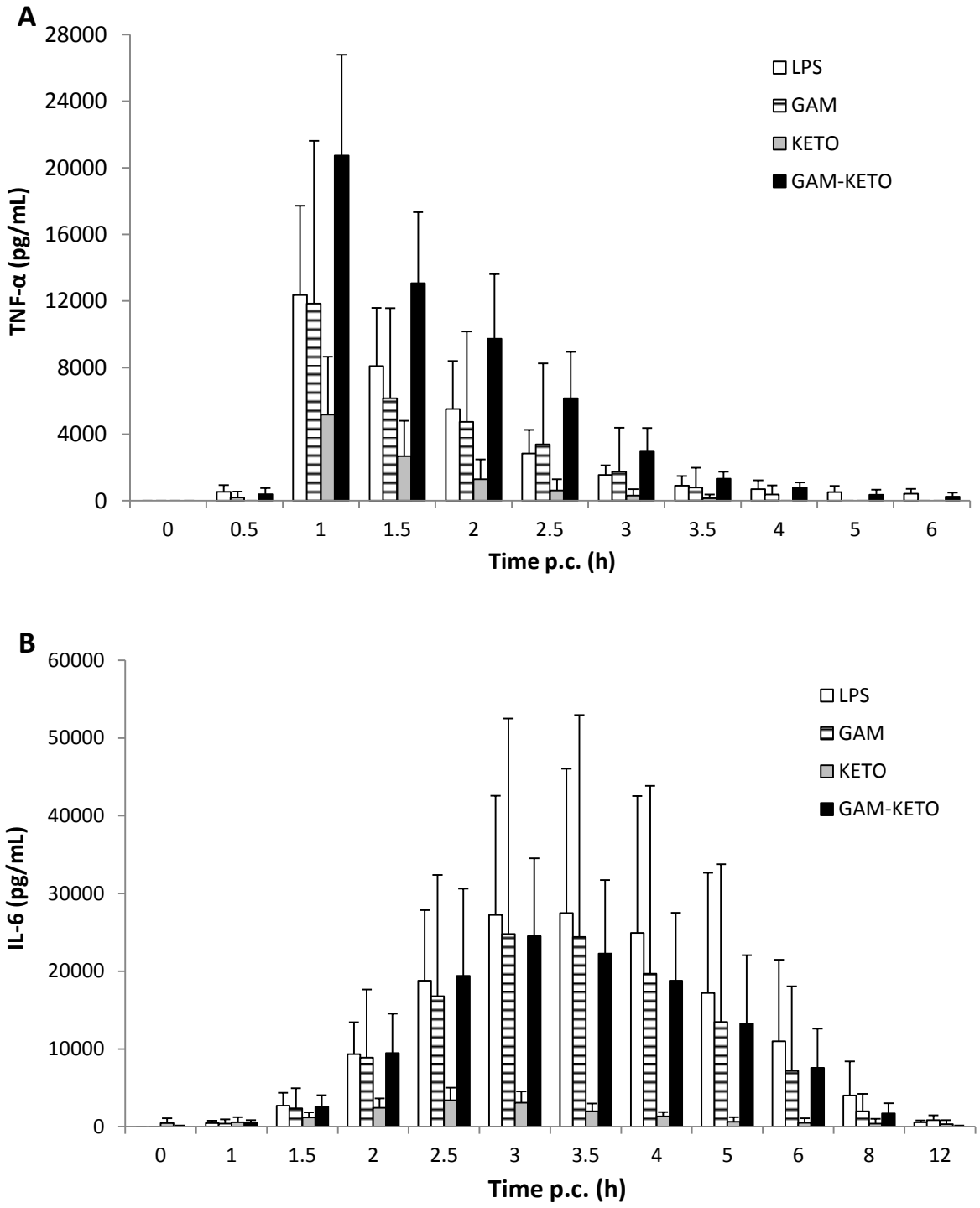

Figure 3.2.5. Mean (+SD) plasma concentrations of TNF-α (A) and IL-6 (B) in 4-week-old calves after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 6) or following pre-treatment with gamithromycin (GAM; n = 4), ketoprofen (KETO; n = 4) or the combination of both drugs (GAM-KETO; n = 4).
TNF-α and IL-6 peak concentrations of the excluded calf in the KETO group were 32,844 and 55,290 pg/mL, respectively.
IL-6 peak concentration of the excluded calf in the GAM-KETO group was 110,524 pg/mL.

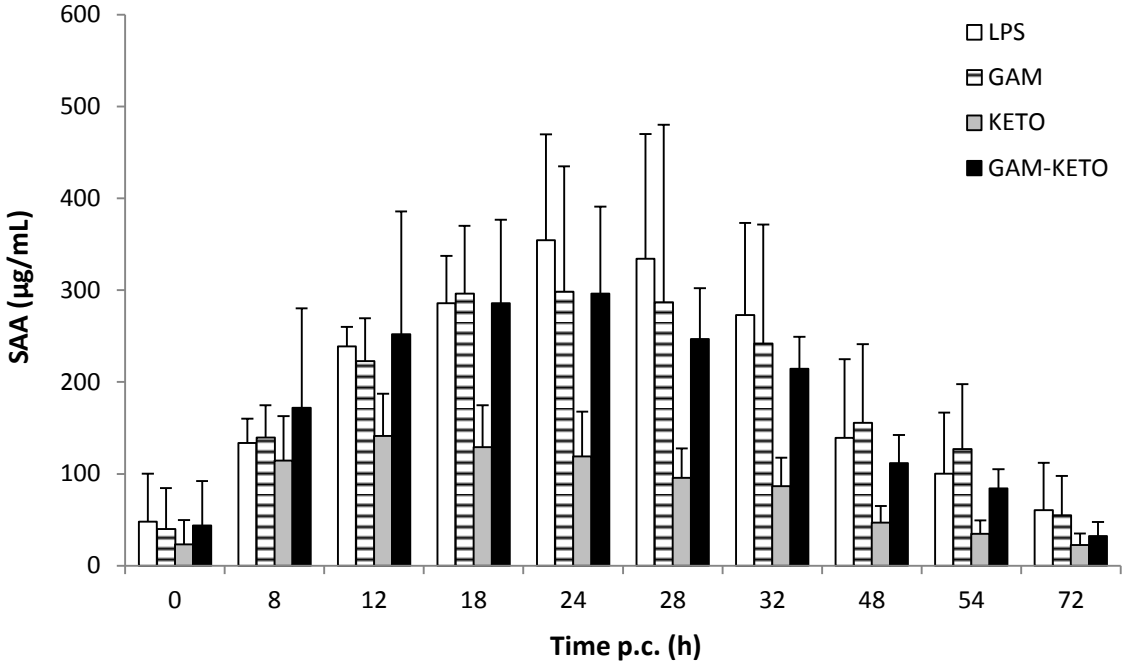

Figure 3.2.6. Mean (+SD) plasma concentrations of SAA in 4-week-old calves after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 4) or following pre-treatment with gamithromycin (GAM; n = 4), ketoprofen (KETO; n = 4) or the combination of both drugs (GAM-KETO; n = 4).

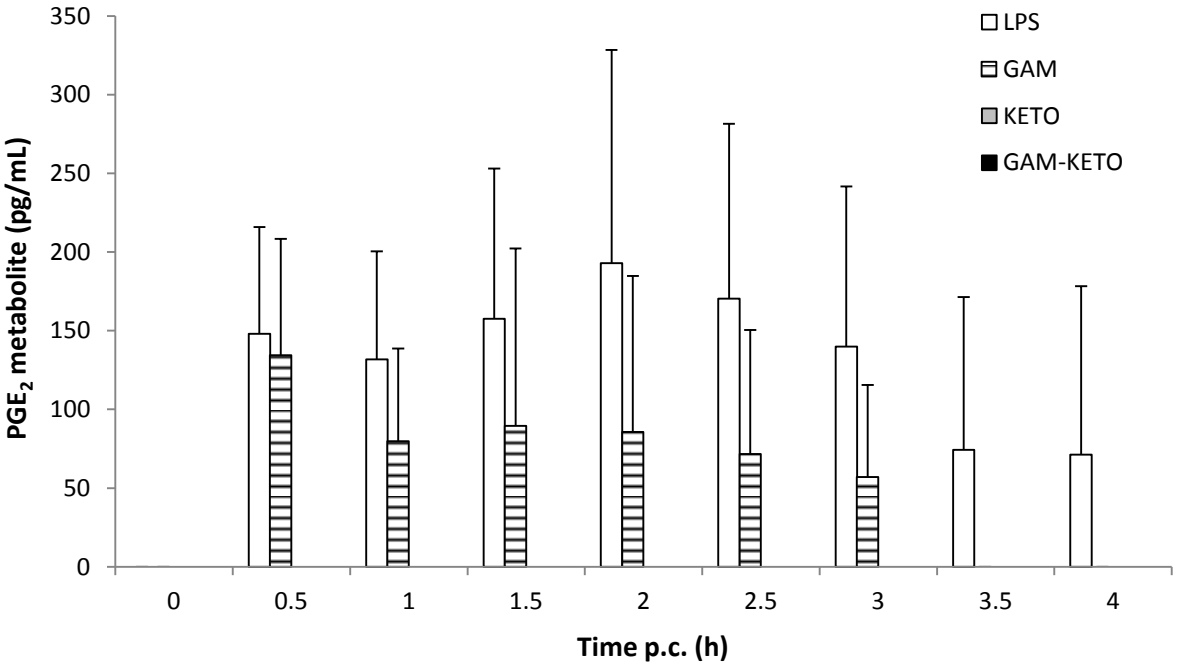

Figure 3.2.7. Mean (+SD) plasma concentrations of PGE_2-*met* in 4-week-old calves after an IV bolus injection of 0.5 µg/kg BW lipopolysaccharide, either not preceded by drug administration (LPS; n = 4) or following pre-treatment with gamithromycin (GAM; n = 4), ketoprofen (KETO; n = 4) or the combination of both drugs (GAM-KETO; n = 4). Only values above the LOQ (50 pg/mL) were included. Ø: no quantifiable concentrations were determined.

4. Discussion

The aim of this study was to investigate the effects of the NSAID KETO on TNF-α, IL-6, SAA, PGE_2 and clinical signs in our previously developed LPS-induced inflammation model in 4-week-old calves. Additionally, the combined administration of KETO and the macrolide GAM was studied to test for possible synergistic and/or additive effects.

Based on the results of previous research, pre-treatment of the calves was considered necessary to induce a maximal immunomodulatory effect (Semrad, 1993a, 1993b). Although such a pre-treatment diverges from clinical settings and it was certainly not the objective to promote prophylactic drug use, this experiment could yield new information regarding the treatment of diseased animals. In this respect, all drugs were administered 1 h prior to LPS challenge, as this interval corresponds to the time of maximum plasma concentration of GAM and the S(+) enantiomer of KETO, which is the pharmacologically active form of this chiral compound (Huang *et al.*, 2010; Plessers *et al.*, 2015a). Both dosages and routes of administration were in accordance to the manufacturers' recommendations (SC and IM for GAM and KETO, respectively).

Despite the advantages of LPS inflammation models regarding standardization and reproducibility, these models know certain limitations in comparison with experimental bacterial infections (Olson et al., 1995; Remick and Ward, 2005). One of these limitations concerns the clinical significance of antimicrobial drugs (i.e. GAM). Nevertheless, the presence of actively invading and growing microorganisms can complicate the interpretation of the effect of immunomodulatory drugs. Endotoxin models can therefore provide a first indication of drug's potential actions, which can subsequently be evaluated in experimental bacterial infection models, and under field conditions.

Pre-treatment with KETO had a remarkable influence on the clinical signs following LPS-challenge. In this respect, all calves in the KETO and GAM-KETO group remained clinically healthy as no severe respiratory distress occurred, nor fever, depression or anorexia. Regarding the pulmonary response, the inhibition of COX and the consequent reduction of arachidonic acid metabolites, including TXA_2, can be suggested (Landoni *et al.*, 1995). Indeed, these inflammatory mediators have been suggested to play a major role in the early

hemodynamic effects after endotoxin administration, including the sharp rise in pulmonary arterial pressure and oedema, and the subsequent development of dyspnea (Tikoff *et al.*, 1966; Esbenshade *et al.*, 1982; Olson and Brown, 1986). The inhibition of plasma concentrations of PGE_2, measured as PGE2-*met*, by KETO was confirmed in our experiment and was additionally reflected by the absence of fever in the KETO and GAM-KETO group. With respect to TNF-α and IL-6, the effect of KETO was not straightforward, as the KETO group clearly demonstrated lower concentrations of these cytokines compared to the GAM-KETO group. As these inconsistencies did not result in a different clinical outcome, the hypothesis from our previous study regarding the involvement of peripherally produced pro-inflammatory cytokines (particularly TNF-α) in depression needs to be reconsidered (Johnson, 2002; Dantzer, 2009; Plessers *et al.*, in preparation).

Compared to the LPS group, pre-treatment with GAM had no significant influence on the clinical signs induced by the LPS challenge, nor on the profiles of the inflammatory mediators. However, it should be mentioned that two calves in the GAM group were euthanized due to severe systemic shock symptoms. Different reasons can be suggested to account for a potential synergistic influence of LPS and GAM in the GAM group and for the elevated cytokine levels in the GAM-KETO group. Although a local swelling can be induced following the SC administration of Zactran®, an additional experiment in three other calves, receiving merely Zactran®, demonstrated that this was not responsible for increased plasma levels of pro-inflammatory cytokines, nor for elevated rectal body temperatures (data not shown). Therefore, a second hypothesis regarding the effect on the lung can be put forward, given that the lung has been ascribed to be the target organ for both LPS and GAM (Tikoff *et al.*, 1966; Huang *et al.*, 2010). In this respect, PIMs, which are constitutively present in cattle, and PIM-related inflammatory mediators might have contributed to the observed differences (Winkler, 1988). Another hypothesis concerns a possible interaction following the intracellular accumulation of both LPS and GAM in phagocytes (Winkler, 1988; Bosnar *et al.*, 2005). More specifically, a bovine *ex vivo* experiment confirmed KETO to reduce TNF-α production in LPS-stimulated whole blood samples, suggesting an inhibitory effect of KETO on transcription factor NF-κB (Tegeder *et al.*, 2001; Donalisio *et al.*, 2013). This reduced nuclear translocation seemed indeed to be present in the KETO group of the current study, while GAM appeared to counteract this influence, resulting in remarkably higher cytokine

levels in the GAM-KETO group. Interestingly, this hypothesis contrasts with earlier studies which suggest a possible suppressive effect of certain macrolides on NF-κB activation (Ianaro *et al.*, 2000; Healy, 2007; Fischer *et al.*, 2011). Due to the limited number of calves in the present experiment, however, further research is needed to confirm these observations.

Overall, the results of the present study suggest a key role for PGE_2 and other prostanoids, both with respect to fever development and depression. Additionally, these clinical signs have been designated to be correlated since depression deteriorates while the rectal body temperature increases (Plessers *et al.*, 2015b). Blatteis *et al.* (2005) extensively reviewed the mechanisms involved in the onset of fever, and questioned the "conventional view", which concerns the significance of pyrogenic cytokines. In this respect, the production of these cytokines and the transduction of their pyrogenic signals into fever-mediating PGE_2 requires more time than the actual start of the febrile response (Conti *et al.*, 2004; Blatteis, 2005; Steiner *et al.*, 2006). Levels of PGE_2-*met* were indeed recorded already at 0.5 h p.c. in the present study. Accordingly, the more recent insights in the course of fever development and sickness behaviour in humans and laboratory animals emphasize the importance of PGE_2, and more specifically, COX-1 induced PGE_2 (Blatteis *et al.*, 2005; Blatteis, 2007; Pecchi, 2009). The latter argumentation has arisen from the need for NF-κB-mediated COX-2 transcription, which again lags the onset of fever (Yamamoto *et al.*, 1995; Blatteis *et al.*, 2005). Moreover, the study of Donalisio *et al.* (2013) reported the preferential activity of KETO to bovine COX-1, the constitutive COX-isoform, mainly involved in physiological processes. This is in contrast with the statement that KETO is a non-selective inhibitor of COX-1 and COX-2, the latter being the inducible COX-isoform, upregulated as part of the inflammatory response (Papich, 2011). It should be mentioned, however, that the distinction between COX-1 and COX-2 functions is not strict, as some crossover occurs (Papich, 2011). From this point of view, the immediate inhibition of COX-1 in PIMs by KETO might be accountable for the reduced pulmonary reaction in the KETO and GAM-KETO group. This hypothesis may also explain the superior effect of KETO on fever development in comparison with DEX, which was studied in the same model (Plessers *et al.*, in preparation). More specifically, the action of corticosteroids requires the *de novo* synthesis of lipocortin in order to cease the formation of arachidonic acid metabolites (Hirata, 1983). Taking the results of these two studies into account, it can be put forward that the administration of

KETO is preferable to DEX in the treatment of calf diseases that are characterized by the systemic involvement of LPS (inclucing pneumonia, septicemia and neonatal diarrhea), given the marked clinical effect of KETO on depression and fever. Furthermore, extrapolation of these findings to the treatment of bacterial infections in practice suggests that the concomitant use of an antimicrobial drug and a NSAID should be considered at all times. In this way, both production results and animal welfare can be enhanced.

In conclusion, the results of the present study demonstrated the capacity of KETO to completely inhibit fever development and depression following an IV endotoxin challenge in 4-week-old calves. This clinical effect was related to the inhibition of PGE_2 by KETO, whereas the involvement of pro-inflammatory cytokines in these clinical signs was shown to be less prominent. GAM, on the other hand, had no influence, neither clinically nor regarding the studied inflammatory mediators. Furthermore, the combination treatment provided no synergistic or additive effects in this model.

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GENERAL DISCUSSION

Modulation of the innate immune response by drugs is the challenge of this doctoral thesis. In this context, the term immunomodulation differs from immunosuppression or anti-inflammation, as it refers to the modification of the endogenous immune responses without impairing the normal immune or inflammatory response to defend against an infection (Kanoh and Rubin, 2010). Accordingly, the following parts of the innate immune response are frequently studied in *in vitro*, *ex vivo* and *in vivo* experiments: phagocytosis, respiratory burst, transcription and release of pro-inflammatory cytokines, chemotaxis and neutrophil apoptosis. Particularly the discovery that certain antimicrobial drugs exert immunomodulatory effects – in addition to their antimicrobial actions – has resulted in an enhanced interest in human medicine. In veterinary medicine, on the other hand, this research field is still in its infancy.

In vivo bovine LPS inflammation models can be applied to study the immunomodulatory properties of e.g. antibiotics, NSAIDs and corticosteroids. More specifically, these models induce a marked acute-phase response in the absence of actively invading and growing microorganisms, which can complicate the interpretation of the drug effect. The results of such studies can subsequently contribute to the development of improved treatment strategies, including superior combination therapies.

Based on these main principles, this doctoral thesis intended to extend the current knowledge on immunomodulatory drugs in bovine veterinary medicine by means of an *in vivo* LPS-challenge model. Figure Ia and Ib give an overview of the main methodologies and achievements of this work, respectively. Figure Ib is based on all calves included in the different animal experiments, by combining the results reported in the different chapters (LPS group: Chapter 1 and Chapter 3.2; GAM group: Chapter 3.1 and 3.2). The challenges that came across during this thesis will be discussed in the following sections, as well as some future prospects for this research area.

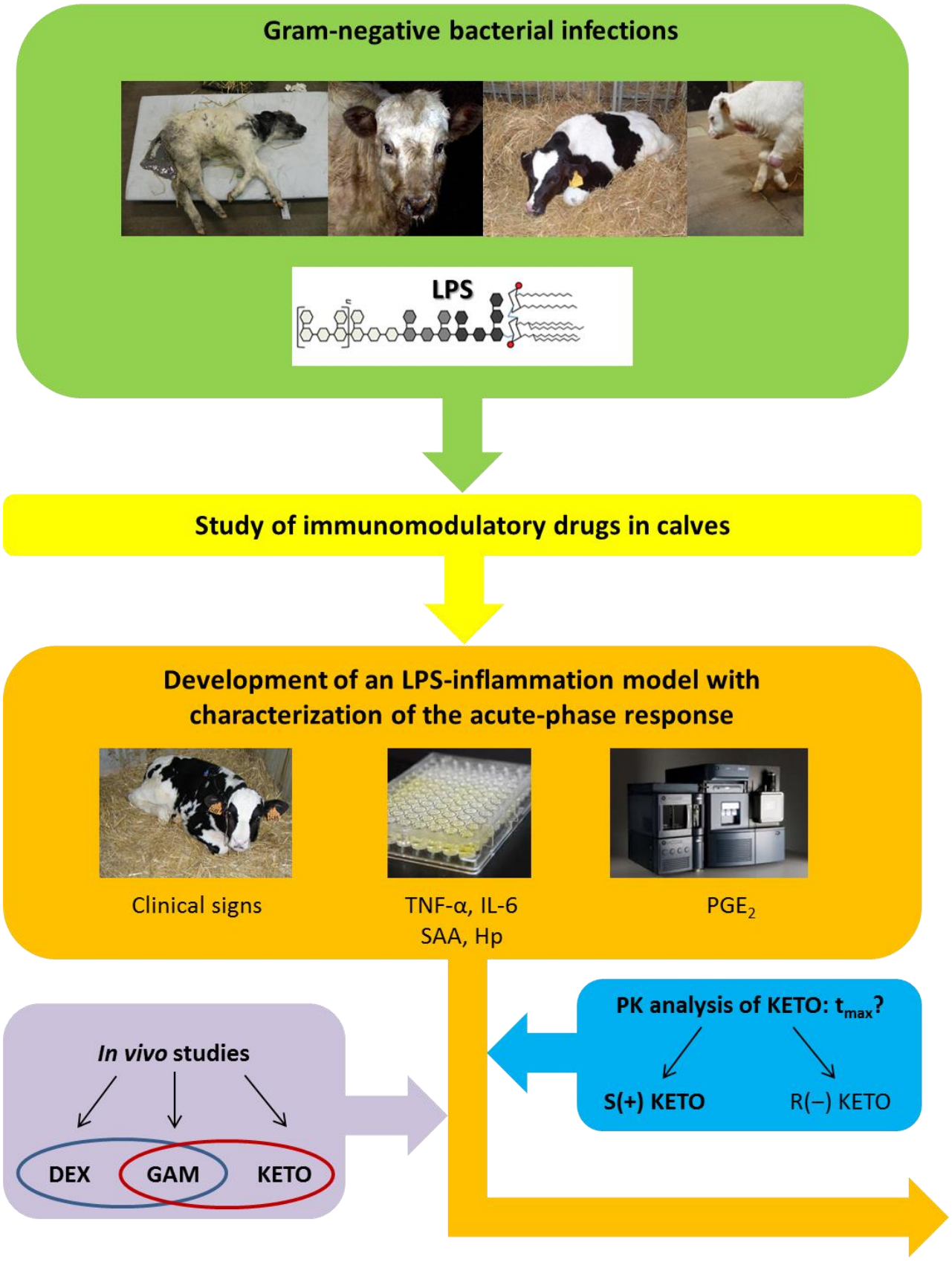

Figure Ia. Overview of the main methodologies of the present doctoral thesis

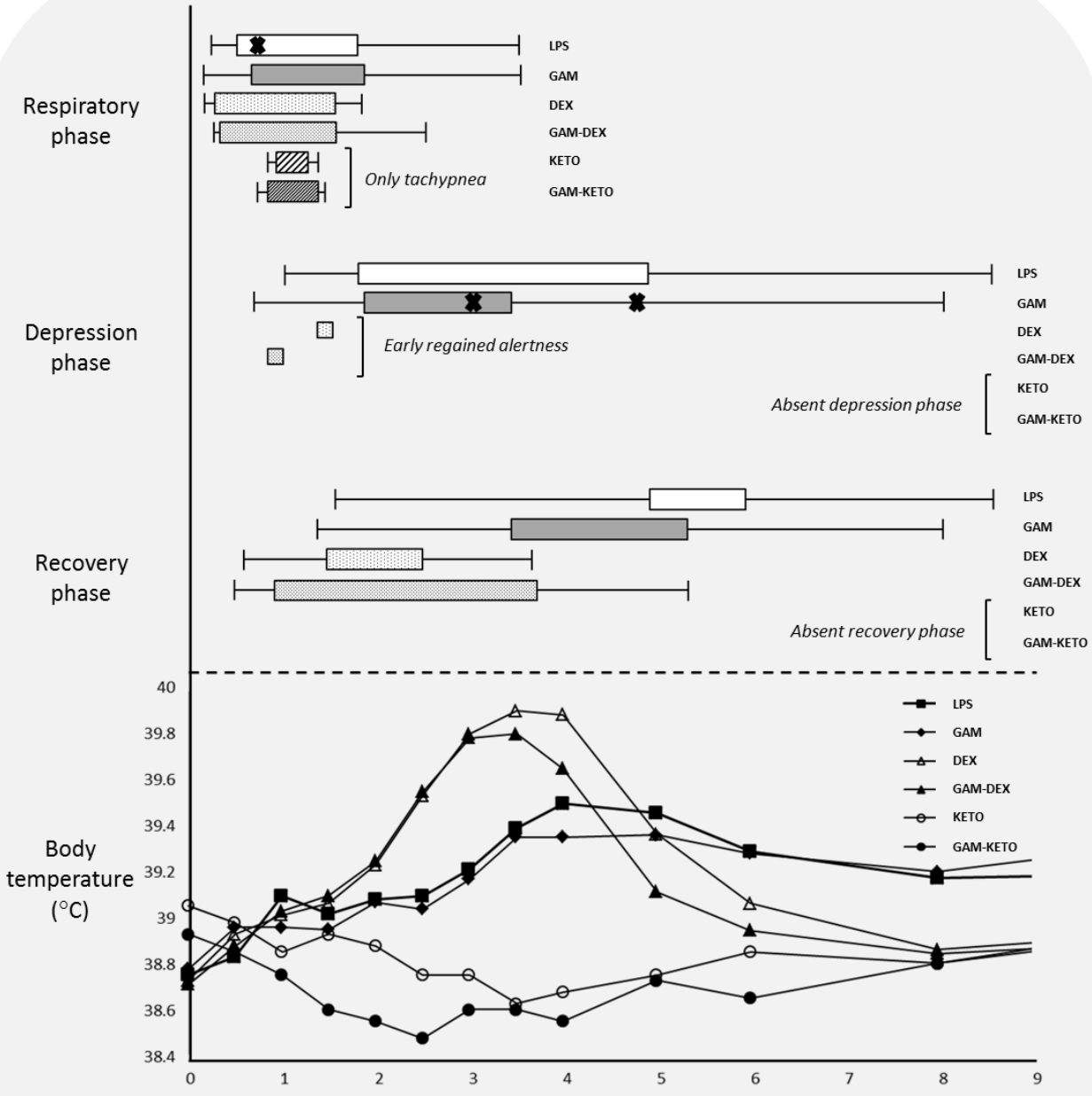

Figure Ib. Overview of the main results of the present doctoral thesis

Development of a standardized and reproducible LPS-inflammation model

In order to mimic the dynamics and multiple interactions that occur during an inflammatory response, *in vivo* experiments can provide more reliable information than *in vitro* studies with various experimental designs (Labro, 1993). Nevertheless, prior to the application of an inflammation model for the pharmacodynamic study of immunomodulary drugs, certain requirements have to be met. In this respect, it is important to elicit a considerable effect on all inflammatory parameters (clinical signs, cytokines, acute-phase proteins and eicosanoids), without inducing (lethal) shock.

It was previously clearly demonstrated that *E. coli* LPS is the most frequently used type of endotoxin to induce an inflammatory response. Additionally, a considerably high reproducibility among studies was found with respect to cytokine release following the administration of this type of LPS. Based on these conclusions, *E. coli* LPS was selected to elicit an acute-phase response in the present work. The use of other types of LPS, on the other hand, can result in a slightly altered clinical outcome. Horadagoda *et al.* (2002), for instance, used *P. multocida* LPS in buffalo calves. This challenge resulted in the rapid onset of clinical signs, characterized by dullness, sternal recumbency, elevated rectal temperatures and dyspnea. Additionally, excessive salivation was induced by this challenge, which is an important symptom of haemorrhagic septicemia caused by *P. multocida* serotype B:2 in buffalos (Horadagoda *et al.,* 2002). These authors also reported rather high inter-animal variations with respect to the clinical LPS response, which might be related to the rather low dose of endotoxin (1 µg/kg BW) in this animal species. Regarding the release of TNF-α, on the other hand, peak levels were reached simultaneously with *E. coli* LPS challenges (1-2 h p.c.) (Horadagoda *et al.*, 2002).

Regarding the age of the calves in the present work, young calves were selected to avoid an extensive anamnesis with respect to Gram-negative bacteria. Nevertheless, studies in neonatal calves were not straightforward concerning fever development (Adams *et al.*, 1990; Gerros *et al.*, 1993; Rose and Semrad, 1992; Semrad *et al.*, 1993). From this point of view, 3-week-old calves were obtained, that were conventionally reared on the farm of origin.

Since calves are extremely sensitive to endotoxin, both the LPS serotype (*E. coli* serotype O111:B4) and dose (0.5 µg/kg BW or 500 units/kg BW) were carefully selected (Michaels and Banks, 1988). Additionally, the clinical condition of the calves at the start of the experiment was considered to be highly important for this type of studies. In this respect, the different origin of the calves and their relatively young age at arrival (3 weeks) might play an interfering role. In order to minimize possible influences, all calves received two preventive treatments, i.e. paromomycin sulphate on the farm of origin and enrofloxacin upon arrival. Interference with the outcome of the experiment could be excluded, as paromomycin sulphate is poorly absorbed after oral administration and the plasma half-life of elimination of enrofloxacin is rather fast in calves (6.8 ± 1.2 h following 12.5 mg/kg BW SC) (EMA, 2000; Davis *et al.*, 2007). Despite the paromomycin treatment, *Cryptosporidium parvum* antigen was still detected in the faeces of a number of calves. Certain calves indeed showed an altered faecal consistency during the acclimatization period, yet remained clinically healthy throughout this period. Furthermore, these animals only received alternating liquid electrolyte therapy until normal faecal consistency was regained. The single enrofloxacin treatment at the start of the 1-week acclimatization period, on the other hand, was found to be highly effective as none of the calves developed respiratory symptoms before the start of the experiment. Still, one out of 10 calves mentioned in the first chapter of this thesis demonstrated severe shock symptoms following the LPS challenge, after which euthanasia was carried out, emphasizing the sensibility of calves to endotoxin.

In order to limit stress and discomfort during the frequent blood sampling, an indwelling catheter was placed in the jugular vein of the calves, at least 12 h prior to the start of the experiment. Additionally, calves were habituated to human presence and contact, as well as to experimental manipulations during the acclimatization period.

Based on the results of the present work, a chronological overview of the appearance of clinical signs and inflammatory mediators following an LPS challenge in 4-week-old calves is provided in Figure II. With the exception of IL-1β, all studied parameters altered significantly in response to the administration of endotoxin.

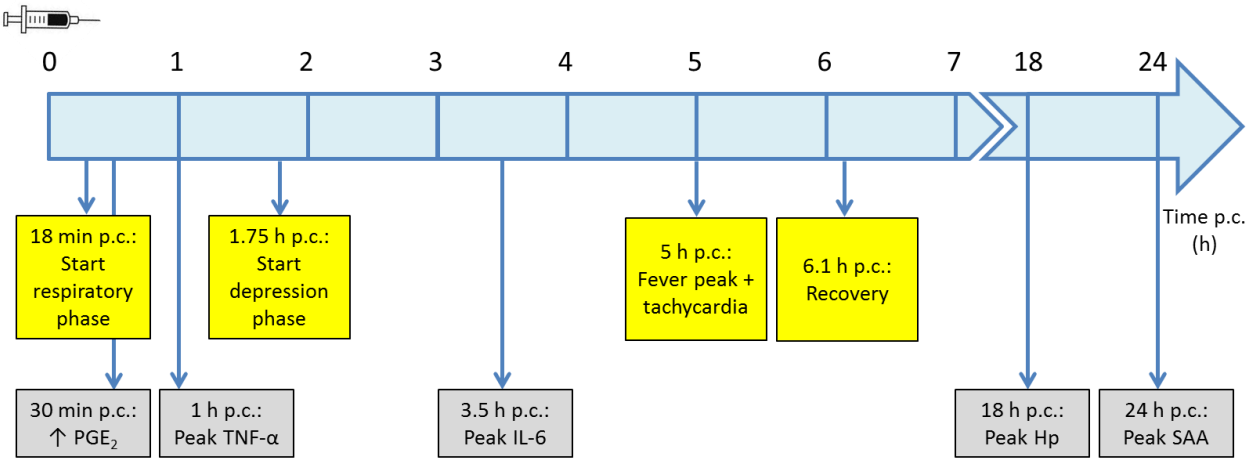

Figure II. Chronological overview of the appearance of clinical signs and inflammatory mediators following an intravenous LPS challenge in 4-week-old calves: results of the present work (p.c.: post LPS challenge)

As discussed in Chapter 1, these observations fairly correspond to the findings of other research groups. The developed model was therefore considered as appropriate with respect to the postulated aims. Nevertheless, it should be remarked that certain inter-animal variations in the different studied parameters were rather high, resulting in substantial standard deviations. Such variations were also reported by Jacobsen *et al.* (2005) in cows. With respect to the course of the rectal body temperature in Chapter 1, a distinction was made between early and delayed responding calves (fever peak ≤ 5 h or ≥ 6 h p.c.). As discussed previously, a pre-shock effect was hypothesized, with the simultaneous occurrence of tachycardia (Lohuis *et al.*, 1988). Surprisingly, these two trends did not continue toward cytokine levels. The involvement of other mediators, including eicosanoids and NO, can therefore be suggested (Salvemini *et al.*, 1990). Regarding other examples of individual variation, one calf in Chapter 1 recovered remarkably fast from respiratory distress and depression. In this respect, tolerance to LPS might have been of importance. LPS tolerance refers to the temporally defined reduction in the intensity of the inflammatory response mediators and clinical signs following repeated exposures to LPS (Lehner and Hartung, 2002; Wang *et al.*, 2003). As no anamnesis on the calves was available upon arrival, the respective calf might indeed have overcome a Gram-negative bacterial infection at the farm of origin. Bieniek *et al.* (1998) summarized the knowledge on this transitory

unresponsiveness to endotoxin, which includes an early and a late tolerance. In this respect, the early tolerance develops 1 h after the first exposure to LPS, is mediated by cytokines, and is associated with a diminished capacity to produce cytokines. The late tolerance, on the other hand, is long-lasting, antibody-dependent and specific for the initially administered type of endotoxin. Nevertheless, the experiment of Bieniek *et al.* (1998) failed to induce tolerance to a second injection of LPS following a 7-day interval in 3-week-old calves. More specifically, the pyrogenic response and heart rates were not influenced. The release of TNF-α, on the other hand, was significantly inhibited in comparison with the first challenge. From this result, the authors concluded that the rectal body temperature was not correlated to TNF-α production. Paradoxically, Elsasser *et al.* (2005) applied two subsequent LPS-challenges to discriminate between normal or tolerant calves, and "hyperresponders". These genetically burdened hyperresponders failed to develop tolerance to repeated LPS challenges, which was evident by the magnitude of TNF-α concentrations and the prolonged severity of pathological conditions. The excessive cytokine release in two calves in Chapter 3.2 (outliers in the KETO and GAM-KETO group) might therefore be attributed to hyperresponsiveness. However, due to the significant effect of the NSAID, the impact on the clinical signs in these suspected hyperresponders could not be evaluated. Regarding the non-responding calf in Chapter 3.2 (GAM-KETO group: neither clinically, nor regarding the studied inflammatory mediators), on the other hand, LPS tolerance might be too straightforward. In this respect, mutations in TLR4 can be hypothesized. Such mutations have been previously associated with endotoxin hyporesponsiveness in humans (Arbour *et al.*, 2000). Also in cattle, TLR mutations were linked to natural resistance to *Mycobacterium avium* subsp. *paratuberculosis* infections (Mucha *et al.*, 2009).

Despite the unpredictable individual variations in the response to an LPS-challenge, the developed model can be applied with respect to the study of immunomodulatory drugs. After all, bovine practitioners are confronted with such variations as well. It should be kept in mind, however, that despite the advantages regarding standardization and reproducibility, the endotoxin model knows certain limitations in comparison with an experimental bacterial infection. One of these limitations concerns the clinical significance of antimicrobial drugs, as was observed in Chapter 3 for gamithromycin.

The fever-and-depression problem

There is disagreement between bovine practitioners concerning the earliest sign of clinical entities like bovine respiratory disease. In this respect, reports on the order of appearance of an increased respiratory rate, coughing, fever and depression are not straightforward (Apley, 2006; Lorenz *et al.*, 2011). Nevertheless, such information is highly important in the development of clinical scoring systems for the early detection of illness, as the latter allows a more effective drug use and a faster recovery. Apley (2006) even suggests that treatment should be initiated on recognition of depression with undifferentiated fever. Accordingly, important signs in the detection of depression are reduced self-grooming, appetite and rumination, as well as an increased time spent lying and standing inactive (Borderas *et al.*, 2008). Johnson (2002) reviewed depression – and sickness behaviour in general – as part of a motivational state (Fig. III). More specifically, this refers to a well-organized adaptive response by the host animal, designed to enhance disease resistance and facilitate recovery.

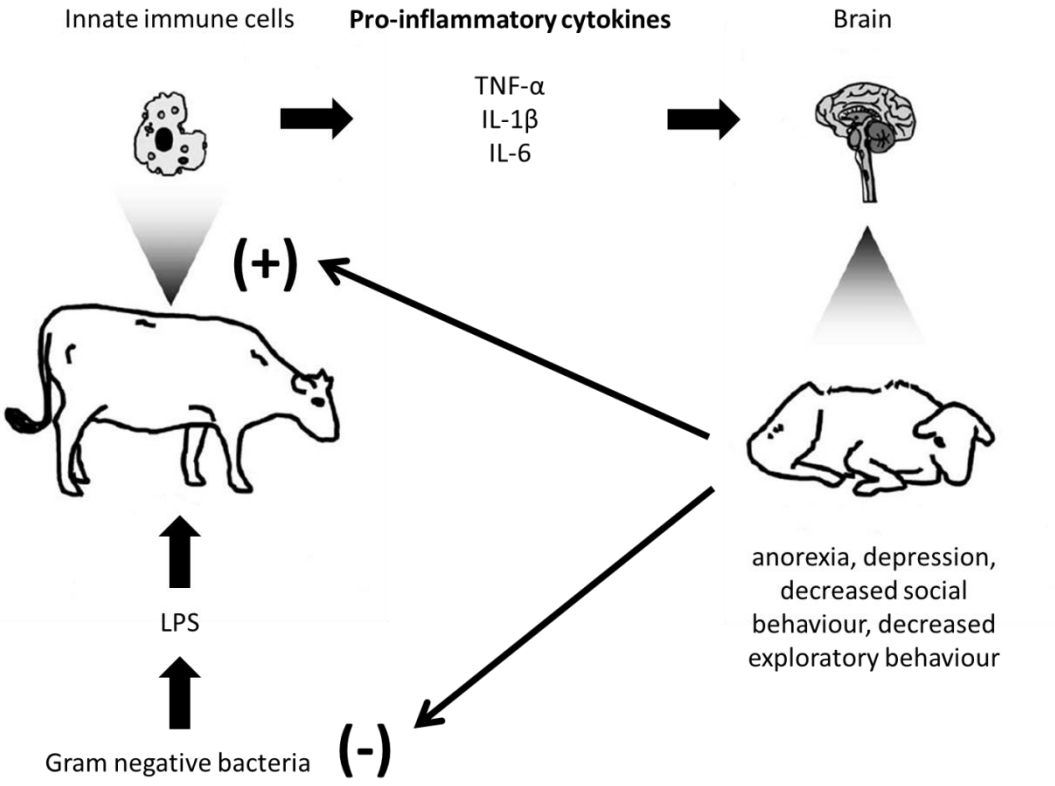

Figure III. Sickness behaviour in cattle (adapted from Johnson, 2002)

From the present work, it can be concluded that depression deteriorates while fever develops, and that alertness is gradually regained once the maximal rectal body temperature is passed. This conclusion emphasizes the importance of depression detection, and consequently supports the view of Apley (2006).

Selection of drugs to be tested in the inflammation model

Based on the literature overview on the immunomodulatory properties of drugs in bovine *in vitro*, *ex vivo* and *in vivo* experiments, three drugs were selected for further *in vivo* studies.

With respect to an **antimicrobial drug**, the choice for a macrolide was the most obvious, relying on the accumulating evidence from human medicine. Zarogoulidis *et al.* (2012), for instance, reviewed the clinical application of macrolides in respiratory diseases in humans, such as chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis. As mainly 14- and 15-membered macrolides have been suggested to exert immunomodulatory properties, the 15-membered semi-synthetic macrolide gamithromycin was selected for the present work (Kanoh and Rubin, 2010). This relatively recently developed azalide is only registered for use as a single SC administration in view of the treatment of bovine respiratory disease (Huang *et al.*, 2010). Although no immunomodulatory effects were observed in our inflammation model (Chapter 3), a beneficial influence of this antibiotic, or other macrolides, in a bacterial infection model would be more likely. Moreover, immunomodulatory effects of macrolides have been reported to be time- and dose-dependent (Buret, 2010).

The **NSAID** ketoprofen was selected for its beneficial effect on TNF-α in the *ex vivo* experiment of Donalisio *et al.* (2013) and on the clinical score following an IV endotoxin challenge (Semrad, 1993a). Due to the direct inhibition of COX by ketoprofen, the enzymatic production of prostanoids ceases. This block explains the anti-inflammatory and antipyretic properties of this drug, which were clearly demonstrated in the present work (Chapter 3.2). With respect to its immunomodulatory properties, ketoprofen reduced the levels of TNF-α

and IL-6 following the LPS challenge (KETO-group in Chapter 3.2), which indeed corresponds to the reports of Donalisio *et al.* (2013).

The **corticosteroid** dexamethasone was included as a positive control immunomodulatory drug in the present work. As described in the general introduction, this choice was based on the reported results from several bovine studies (Ohtsuka *et al.*, 1997a; Kiku *et al.*, 2002; Malazdrewich *et al.*, 2004a). Dexamethasone indeed confirmed its immunomodulatory capacities with respect to the release of pro-inflammatory cytokines, while its clinical effect following an IV endotoxin challenge was rather limited (Chapter 3.1). The latter observation was somewhat surprising, as other studies demonstrated a clear effect of this corticosteroid on the cardiopulmonary response and fever development during an inflammatory response (Olson and Brown, 1986; Lohuis *et al.*, 1989). Additionally, it must be mentioned that in the animal trial described in Chapter 3.1 no extra positive control group was included, receiving merely LPS. Nevertheless, the calves of the LPS-group in Chapter 1 and Chapter 3.2 showed relatively similar profiles of all studied parameters, demonstrating the reproducibility of the developed inflammation model.

Selection of the time of drug administration

It was concluded that the administration of the drug after the challenge could not significantly influence the inflammatory response (Semrad, 1993a, 1993b). In this respect, pre-treatment of the calves was considered necessary to induce a maximal immunomodulatory effect. Although such a pre-treatment diverges from clinical settings and although it was certainly not the objective to promote prophylactic drug use, this experimental design could yield new information with respect to the treatment of diseased animals.

The time of pre-treatment with **gamithromycin and ketoprofen** was based on their PK properties. More specifically, the time of maximum plasma concentration of the drugs (t_{max}) was taken into account as a maximal effect of the drugs was expected at this time. As PK data were lacking for the racemic mixture of ketoprofen following the frequently applied IM administration, an additional study was performed in calves (Chapter 2). In this respect,

plasma concentrations of the R(–) and S(+) enantiomer were quantified separately, since the S(+) enantiomer is the pharmacologically active form of this chiral compound (Cabré *et al.*, 1998). Based on the results of the latter study (t_{max} of S(+) ketoprofen at 0.98 ± 0.18 h) and the study of Huang *et al.* (2010) concerning gamithromycin (t_{max} at 1.0 ± 0 h), the interval to the LPS-challenge was set at 1 h for both drugs. The distinct results from Chapter 3.2 demonstrate that this interval was appropriately chosen for ketoprofen: cytokine release was reduced and the clinical effect of the LPS challenge was completely blocked following pre-treatment with this NSAID. As no influence of gamithromycin on the immune response was observed following LPS administration at t_{max}, both the applied interval and dose can be questioned, due to the time- and dose dependency of the immunomodulatory effects of macrolides (Buret, 2010).

The administration of **dexamethasone** 1 h prior to the challenge was based on the study of Ohtsuka *et al.* (1997a) in calves. Similarly to the findings of these authors, a clear influence on TNF-α release was found in the present work (Chapter 3.1). The selected interval can therefore be considered as suitable for this positive control immunomodulatory drug. Nevertheless, one hour was most likely insufficient to induce high levels of lipocortin. This protein is required for the inhibition of PLA_2, which in turn ceases the formation of arachidonic acid metabolites. From this point of view, the experimental design from Olson and Brown (1986), with two pre-treatments (18 and 1 h prior to the LPS challenge), may have exerted a more pronounced influence on the pulmonary and febrile response.

The potential contradictory effect of gamithromycin

The animal experiments revealed a notable effect of gamithromycin on the clinical outcome of an endotoxin challenge. In Chapter 3.1, this was related to the delayed responding fever profile (peak at 12 h p.c. in comparison with the average at 5 h p.c.), while in Chapter 3.2, two calves were euthanized at 3 and 4.75 h p.c. These observations might be both related to the induction of shock. Also in pigs, the administration of GAM prior to an IV LPS challenge has been described to result in a delayed recovery (Wyns *et al.*, 2015a). The effects of gamithromycin observed in the present work were not reflected by cytokine

responses, as no significant differences could be observed between the GAM and LPS groups. Nevertheless, the group that received both gamithromycin and ketoprofen (Chapter 3.2) demonstrated clearly higher concentrations of TNF-α and IL-6 compared to the group that received merely the NSAID. The beneficial clinical effect of the ketoprofen-treatment, on the other hand, was not affected in this combination group. Conversely, such an effect on cytokine levels was not present following the combined administration of gamithromycin and dexamethasone (Chapter 3.1), suggesting that the corticosteroid-related repression of NF-κB is more pronounced compared to the NSAID.

As discussed in Chapter 3.2, different reasons can be suggested for the remarkable influence of gamithromycin. An effect of the formulation was ruled out following an additional experiment, whereas the role of PIMs and the consequences of the intracellular accumulation of both LPS and gamithromycin in phagocytes need further elucidation. The involvement of PIM-related inflammatory mediators – including TXA_2 – has been suggested to contribute to the more severe symptoms in the gamithromycin-treated group, particularly since the lung has been ascribed to be the target organ for both LPS and gamithromycin (Tikoff *et al.*, 1996; Huang *et al.*, 2010). Furthermore, due to the high volume of distribution of gamithromycin (24.9 L/kg), high intracellular concentrations of this macrolide are reached following parenteral administration (Winkler, 1988; Bosnar *et al.*, 2005; Huang *et al.*, 2010). In this respect, it can be hypothesized that the presence of both gamithromycin and LPS in phagocytes results in an interaction. Moreover, Labro (1993) stated that intracellular drug concentrations may indeed alter certain phagocyte functions. It should be remarked, however, that the number of animals in the present work was rather limited, and that additional research is needed to confirm the reported observations.

Cytokines or PGE_2?

With respect to the involvement of cytokines and PGE_2 in sickness behaviour and fever development, several authors hold on to the unequivocal role of pro-inflammatory cytokines ("the conventional view"), while others suggest a more important contribution of PGE_2 (Johnson, 2002; Blatteis *et al.*, 2005; Dantzer, 2009; Pecchi *et al.*, 2009). As previously

discussed, the results of the *in vivo* studies (Chapter 3) rather support the idea of the major involvement of PGE_2, in both sickness behaviour and the onset of fever. Initially, in Chapter 3.1, the association between depression and cytokines was hypothesized, since pre-treatment with dexamethasone resulted in a faster recovery from the challenge and remarkably lower levels of TNF-α and IL-6. The results of Chapter 3.2 subsequently conflicted with this hypothesis as the calves in the GAM-KETO group demonstrated no behavioural changes, despite high levels of the pro-inflammatory cytokines. The importance of the inhibition of PGE_2 production – and other prostanoids – could therefore be put forward with respect to reduced sickness behaviour (Pecchi *et al.*, 2009). Teeling *et al.* (2007) also demonstrated a key role for prostaglandins in this context, as low grade inflammation was observed to impact on the brain, independently of peripheral cytokine production.

The role of PGE_2 in fever development was already proposed in Chapter 3.1. Since the dexamethasone-induced inhibition of cytokines had no prominent influence on the febrile response following the LPS challenge, the involvement of prostanoids was suggested, based on the rather slow action of corticosteroids. However, as their analysis requires a specific sampling protocol (i.e. addition of indomethacin in order to prevent *ex vivo* artefactual eicosanoid generation), these inflammatory mediators could not be determined in Chapter 3.1. In Chapter 3.2, the hypothesis concerning the involvement of PGE_2 in fever was then confirmed by the direct inhibition of COX by ketoprofen. In this respect, the calves in the KETO and GAM-KETO group demonstrated no quantifiable PGE_2 concentrations, nor any changes regarding the course of the rectal body temperature. The inhibition of PGE_2 synthesis by ketoprofen in inflammatory exudate was also demonstrated in the study of Landoni and Lees (1995b), following the injection of carrageenan in SC implanted tissue cages in calves.

The important role of PGE_2 can also be established from the chronological overview of the appearance of clinical mediators following the challenge (Fig. II). More specifically, high levels of PGE_2 were already detected 0.5 h p.c., whereas concentrations of TNF-α and IL-6 were still relatively basal or absent at this time. The early release of PGE_2 was clearly associated with the pulmonary response, which started on average 18 min p.c., and which was linked to the presence of LPS-clearing PIMs in the lung (Winkler, 1988). Additionally, the

onset of the febrile response preceded the peak of the pyrogenic cytokine IL-6 (at 3.5 h p.c.). As previously discussed, the occurrence of depression and fever are thought to be correlated, suggesting a key role for PGE_2 and other prostanoids in sickness behaviour as well. Overall, these observations emphasize that the "conventional view" with respect to fever development and clinical signs needs to be reconsidered. The quantification of prostanoids in bovine plasma can definitely contribute to this research. Although the development of a specific and accurate ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS) method is quite challenging, such a method can provide several advantages compared to ELISAs. More specifically, the specificity of ELISAs in the analysis of eicosanoids can be questioned, due to the abundant presence of isomers, which can possibly cross-react (Mesaros *et al.*, 2009). An LC-MS/MS method, on the other hand, is characterized by a very high specificity and sensitivity, which allows the quantification of rather low concentrations of the eicosanoids of interest.

NSAID or corticosteroid?

The choice between a NSAID and a corticosteroid as an ancillary therapy in the treatment of acute bacterial infections in cattle remains a vexed question. Both practitioners and farmers aim at a fast clinical response, rather than taking the side effects of corticosteroids in account. Nevertheless, the results of the present work demonstrate that ketoprofen is capable to exert an outstanding effect on the clinical condition of the animal, with complete suppression of depression and fever, while the influence of dexamethasone was rather limited (Chapter 3). A similar conclusion was formulated by Bednarek *et al.* (2003) following the comparative study of meloxicam and flumethasone – both in combination with oxytetracycline – for the treatment of enzootic bronchopneumonia in calves. More specifically, a significantly faster improvement of the clinical score was observed following the treatment with meloxicam. However, in contrast to the results of the present work, both meloxicam and flumethasone reduced the mean rectal body temperature in these naturally infected calves. It should be mentioned that the body temperature was only determined 24 h after the treatment with the anti-inflammatory drugs, and that higher lipocortin levels were presumably reached during this prolonged time interval, in comparison with the

treatment 1 h prior to LPS. Also in the meeting abstract of Olaerts *et al.* (1995), a significant effect on the febrile response by ketoprofen as well as dexamethasone – without the concurrent administration of an antimicrobial drug – was reported. Nevertheless, in this model of experimentally induced pasteurellosis in calves, dexamethasone could not inhibit hyperpnea and a higher frequency of macroscopic lung lesions was observed following the treatment with this corticosteroid. From this point of view, these authors suggested ketoprofen to be more appropriate in the modulation of lung inflammation. Indeed, the attenuation of pulmonary inflammation can prevent severe dysfunctions and lesions, which can be responsible for irreversible lung damage or death (Lekeux, 2006).

Whereas experimental and field tests with respect to corticosteroids as (ancillary) therapy in the treatment of acute inflammatory processes have yielded conflicting results, the conclusions of such studies using NSAIDs were notably more straightforward. Christie *et al.* (1977), for instance, reported a poorer response to treatment, a higher relapse rate and a slower recovery from disease when dexamethasone was used as ancillary therapy for bovine respiratory disease treatment. Conversely, Sustronck *et al.* (1997) found a superior effect of the combination of flumethasone and sodium ceftiofur to the antibiotic alone in experimental pasteurellosis in calves. This was characterized by a faster recovery and a decreased mortality rate. Finally, Espinasse *et al.* (1992) could not demonstrate a beneficial effect from the additional administration of prednisolone compared to the treatment with merely ceftiofur in experimental *M. haemolytica* bronchopneumonia in calves. With respect to NSAIDs, on the other hand, all studied drugs (carprofen, flunixin meglumine, ketoprofen, meloxicam and tolfenamic acid) have been shown to be effective as ancillary therapy in the treatment of bovine respiratory disease (Deleforge *et al.*, 1994; Balmer *et al.*, 1997; Lockwood *et al.*, 2003; Elitok and Elitok, 2004; Friton *et al.*, 2005; Guzel *et al.*, 2010). Even the administration of merely carprofen significantly decreased clinical scores and pulmonary lesions following an experimental infection with *M. haemolytica* (Wallemacq *et al.*, 2007).

Based on the results of the present work and the findings in literature, it can be concluded that NSAIDs can be preferred to corticosteroids as an ancillary therapy, particularly in the treatment of calf diseases that are characterized by the systemic involvement of LPS. The NSAID does not only improve animal welfare, but also exerts

beneficial long-term effects regarding production results. In this respect, Friton *et al.* (2005) reported a significantly higher mean daily weight gain and mean carcass weight following the concomitant administration of meloxicam and oxytetracycline for the treatment of bovine respiratory disease, as well as significantly less lung lesions at slaughter. Nevertheless, the risk for (gastrointestinal) side effects of non-selective NSAIDs should be taken into account as well. The combined administration of an NSAID and a corticosteroid, on the other hand, should be avoided at all times, as this combination results in a higher incidence of gastroenteritis, gastrointestinal ulcerations and gastrointestinal perforations compared to NSAIDs (Curry *et al.*, 2005). Moreover, corticosteroids have been associated with illegal growth promoting effects.

Perspectives on the use of immunomodulatory drugs in cattle practice

As previously reported, data on the immunomodulatory properties from bovine *in vivo* studies are much scarcer than those from *in vitro* studies. The extrapolation from *in vitro* to *in vivo* situations, however, is not feasible as the results from *in vitro* studies are in some cases inconsistent, and dependent on the experimental protocol. Nevertheless, *in vitro* experiments can provide an indication of certain immunomodulatory actions. In this respect, Fischer *et al.* (2011) studied the effect of tulathromycin on bovine neutrophil apoptosis both *in vitro* and *in vivo*. A significant increase was found in both experiments, although it should be mentioned that the *in vivo* experiment included an experimental infection with *M. haemolytica* in calves. A more recent study of the same authors also demonstrated the inhibition of the chemoattractant LTB_4 by tulathromycin *in vitro* and *in vivo*, the latter in a nonbacterial model of pulmonary inflammation (Fischer *et al.*, 2014). Additionally, bovine studies using tilmicosin and tulathromycin have confirmed the inhibition of PGE_2 by both macrolides *in vitro*, *ex vivo* as well as *in vivo* (Lakritz *et al.*, 2002; Fischer *et al.*, 2014). These results are clearly in accordance with the reports on the immunomodulatory properties of macrolides from human medicine, and emphasize their potential added value in the treatment of diseased animals.

In contrast to the reports of the former bovine studies, gamithromycin exerted no immunomodulatory effects in the inflammation model developed in the present doctoral thesis. In this respect, the reduced nuclear translocation of NF-κB that was observed for tulathromycin did not continue toward gamithromycin, as the release of pro-inflammatory cytokines in LPS-challenged calves was not influenced. Pre-treatment with gamithromycin did not reduce PGE_2 synthesis either, suggesting the absence of an influence of the macrolide on COX expression and/or PLA_2 activity, as described by Fischer *et al.* (2014). Ketoprofen, on the other hand, reduced plasma levels of TNF-α, IL-6 and PGE_2. Also from this point of view, the concomitant use of a NSAID and an antimicrobial drug can be recommended. Nevertheless, this combination did not result in a more pronounced beneficial clinical effect in the inflammation model, compared to the exclusive administration of ketoprofen (Chapter 3.2). On the contrary, higher concentrations of the studied pro-inflammatory cytokines were measured following the combined administration of gamithromycin and ketoprofen, though without affecting the clinical outcome. Levels of PGE_2, on the other hand, remained below the limit of quantification of the UPLC-MS/MS method. Overall, these findings suggest the beneficial effect of the combination of gamithromycin and ketoprofen in experimental bovine bacterial infection models, which should subsequently be studied under field conditions in bacterial diseases.

Extrapolation to other NSAIDs

The distinct influence of ketoprofen on the clinical outcome of an LPS challenge raises the question whether all NSAIDs are capable to exert such an effect. Although the general action of NSAIDs concerns the direct inhibition of COX, certain aspects should be taken into consideration.

As previously reported in this doctoral thesis (Chapter 3.2), the more recent insights with respect to fever development and sickness behaviour have suggested a key role for PGE_2, and more specifically COX-1 induced PGE_2 (Blatteis *et al.*, 2005; Blatteis, 2007; Pecchi, 2009). This constitutive COX-isoform would be responsible for the immediate release of prostanoids in response to the IV administration of endotoxin. The transcription of COX-2,

on the other hand, is upregulated as part of the inflammatory response, and consequently induces a delay regarding the synthesis of PGE_2. This view provides an explanation for the remarkable effect exerted by ketoprofen in the present work: although this NSAID is generally referred to as a non-selective COX-inhibitor, the *ex vivo* study of Donalisio *et al.* (2013) reported a preferential action of ketoprofen to bovine COX-1. In this respect, other NSAIDs showing a preference for bovine COX-1 can be of particular interest. Flunixin meglumine, for instance, can be put forward since the study of Donalisio *et al.* (2013) demonstrated similar effects as observed for ketoprofen: a significant inhibition of TNF-α as well as a preferential activity versus COX-1. Additionally, Semrad (1993a, 1993b) reported a reduction of the clinical score following an *in vivo* LPS-challenge in calves by flunixin meglumine. As mentioned before, however, the risk for gastrointestinal side effects of these COX-1-preferring NSAIDs should be taken into consideration as well.

Also in other animal species, ketoprofen and flunixin meglumine are considered important mainstays in the treatment of endotoxemia (Kelmer, 2009; Peters *et al.*, 2012; Wyns *et al.*, 2015a). Nevertheless, extrapolation of drug effects to other animal species needs to be performed with caution, as several factors can interfere. These factors include anatomical, biochemical and physiological differences, which can result in species-specific pharmacokinetics (PK) and pharmacodynamics (PD) (Lees *et al.*, 2004). More specifically, each of the pharmacokinetic processes (absorption, distribution, metabolism and excretion) can differ between animal species, with variations in drug metabolism being the major factor accounting for species differences in PK and PD (Toutain *et al.*, 2010). In this respect, the production of active or toxic metabolites can result in divergent clinical outcomes. Additionally, a number of side effects of NSAIDs have been demonstrated to be more pronounced in certain species (Rainsford *et al.*, 2003). Regarding oral porcine ketoprofen preparations, for instance, the drug leaflets mention a high risk for gastric ulcers. Accordingly, these porcine preparations were retrieved from the Belgian market.

Besides the formerly mentioned influencing factors, the formulation of the drug can also affect the clinical outcome. Firstly, non-active substances, including excipients, can induce side effects (Golightly *et al.*, 1988). Secondly, as the formulation plays a critical role in drug absorption, it can influence the onset and duration of action of the drug (Grady and

Stahl, 2012). The preparation of dexamethasone used in the present work, for example, was formulated as a phosphate ester prodrug and consequently needs to be hydrolysed after administration, possibly delaying its activity.

Overall, these conclusions suggest that each formulation of each drug should be investigated on a species-by-species basis to guarantee its effective and safe use.

Future perspectives

Based on the results from the present doctoral thesis, the following perspectives can be formulated with respect to the developed *in vivo* inflammation model:

- Enclosure of other eicosanoids in the UPLC-MS/MS method, including TXA_2 and LTB_4. Particularly TXA_2 was frequently mentioned in this work with respect to its contributing role to the pulmonary response following an endotoxin challenge. Additionally, the determination of proresolving lipoxins might be of interest. In cattle, the promoting effect of tulathromycin on lipoxin A_4 release has already been confirmed (Fischer *et al.*, 2014).

- Study of the effect of dexamethasone on PGE_2 levels in the inflammation model following a single treatment as well as following repeated treatments. Knowledge on the profile of this prostanoid could provide valuable information regarding the observed febrile response in dexamethasone-treated calves. However, the specific sampling protocol using indomethacin should be taken into consideration.

- Optimization of the use of PGE_2 and other eicosanoids, determined by the UPLC-MS/MS multi-method, as end-points for efficacy testing of NSAIDs.

- Evaluation of the immunomodulatory properties of other antimicrobial drugs, NSAIDs and corticosteroids in the developed LPS inflammation model. In this respect, the 15-membered macrolide tulathromycin might be of interest, based on the results of Fisher *et al.* (2011, 2013, 2014). Regarding NSAIDs, on the other hand, flunixin meglumine can be put forward for its preferential activity to COX-1 (Donalisio *et al.*, 2013).

- Assessment of the studied drugs in experimental bacterial infection models (including *M. haemolytica* and *P. multocida*), with emphasis on the effect of the concomitant use of antimicrobial drugs and NSAIDs.

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SUMMARY

As part of the outer membrane of Gram-negative bacteria, lipopolysaccharide (LPS) is involved in several clinical entities in cattle, and particularly in calves, including neonatal diarrhea, pneumonia and septicemia. The exogenous administration of LPS has been widely applied in veterinary research to study diverse aspects of the acute-phase response. Moreover, it has been established that such LPS inflammation models can be used to study the immunomodulatory properties of drugs. In this context, immunomodulation refers to the relatively recent research field aiming to modify the innate immune responses to benefit the host in the treatment of diseases. Consequently, immunomodulation does not strictly refer to immunosuppression or anti-inflammation. Studies in humans and laboratory animals demonstrated that certain antimicrobial drugs (including macrolide antibiotics), non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids possess immunomodulatory properties, irrespectively of their direct pharamacodynamic effects. In cattle, on the other hand, reports on this topic are rather limited. Nevertheless, this information can contribute to the development of improved treatment strategies for LPS-associated diseases, resulting in economic, public and animal benefits.

Therefore, the **GENERAL AIM** of this doctoral thesis was to evaluate the immunomodulatory properties of the macrolide antibiotic gamithromycin (GAM), the corticosteroid dexamethasone (DEX) and the NSAID ketoprofen (KETO) alone, as well as their two-drug combinations (GAM-DEX and GAM-KETO) in a standardized LPS inflammation model in calves, with emphasis on the acute-phase response.

In the **GENERAL INTRODUCTION**, an overview of the reported bovine *in vivo* LPS inflammation models was provided with respect to the LPS-induced acute-phase response. More specifically, the release of pro-inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6) and acute-phase proteins (serum amyloid A (SAA) and haptoglobin (Hp)) was highlighted, as well as the clinical signs following the challenge: pulmonary response, fever, tachycardia and behavioural changes including depression and anorexia. Additionally, the results from bovine *in vitro*, *ex vivo* and *in vivo* studies regarding the immunomodulatory properties of antibiotics, NSAIDs and corticosteroids were discussed.

In **CHAPTER 1**, the development and characterization of an LPS inflammation model in 4-week-old calves was described. In this respect, a single intravenous dose of 0.5 µg/kg body weight ultrapure *Escherichia coli* LPS was selected to induce an acute-phase response. This bolus administration resulted in the following clinical signs: tachypnea (on average 20 min post challenge (p.c.)), decubitus (30 min p.c.), general depression (1.75 h p.c.), fever (5 h p.c.) and tachycardia (5 h p.c.). Subsequent to the recovery from respiratory distress, general depression was prominent, which deteriorated when fever increased. Once the maximal rectal body temperature was passed, alertness and appetite were gradually regained. The calves recovered on average within 6 h p.c. With respect to the induced inflammatory mediators, peak plasma concentrations of TNF-α, IL-6, SAA and Hp were detected at 1, 3.5, 24 and 18 h p.c., respectively.

In **CHAPTER 2**, the pharmacokinetic properties of KETO were determined following intramuscular (IM) administration in calves. Due to possible enantioselective disposition kinetics and chiral inversion, the plasma concentrations of the R(–) and S(+) enantiomer were quantified separately. The results demonstrated a distinct predominance of the S(+) enantiomer, as well as significantly different pharmacokinetic parameters between R(–) and S(+) KETO. The time to maximal plasma concentration (t_{max}) of S(+) KETO, the pharmacologically active form of this chiral compound, was found to be 0.98 ± 0.18 h.

In **CHAPTER 3**, the immunomodulatory properties of GAM, DEX and KETO, as well as their two-drug combinations were assessed in our standardized LPS inflammation model. DEX was selected as a positive control immunomodulatory drug. Based on the work of other research groups, pre-treatment was considered necessary in order to elicit a maximal effect of the drug on the immune response. Therefore, all drugs were administered 1 h prior to the LPS challenge, either subcutaneously (GAM) or IM (DEX and KETO). This interval was in accordance with the t_{max} of these drugs in calves. Two studies were performed (**CHAPTER 3.1** and **CHAPTER 3.2**), using a similar study design in both chapters. However, Hp was only included in Chapter 3.1, whereas prostaglandin E_2 (PGE_2) concentrations were only determined in Chapter 3.2.

GAM alone had no significant effect on the production of TNF-α, IL-6, SAA, Hp and PGE_2, nor on the clinical signs induced by the LPS challenge. This was in contrast to previous

reports on other macrolides. DEX, on the other hand, inhibited cytokine levels and resulted in a faster recovery, whereas the onset of respiratory distress and fever were not altered by this corticosteroid. KETO exerted the most pronounced effects in our inflammation model, as the calves remained clinically healthy throughout the experiment: fever, depression and anorexia were completely counteracted. Moreover, cytokine and SAA concentrations were reduced and PGE_2 levels were absent following KETO pre-treatment. Regarding the two-drug combinations, no synergistic or additive effects were observed in this model. Conversely, the GAM-KETO group demonstrated higher cytokine levels compared to the KETO group, though without affecting the clinical outcome.

In **CONCLUSION**, we developed a standardized and reproducible LPS inflammation model in calves to evaluate the immunomodulatory properties of drugs. KETO inhibited all studied aspects of the acute-phase response: cytokine, SAA and PGE_2 levels, fever, depression and anorexia. GAM, on the other hand, had no effect, while DEX exerted an intermediate influence. Overall, these findings suggest that prostaglandins outweigh pro-inflammatory cytokines with respect to LPS-induced sickness behaviour. As a consequence, this doctoral thesis emphasizes the beneficial effect of the concomitant use of an antibiotic and a NSAID in the treatment of acute bacterial infections.

SAMENVATTING

Lipopolysaccharide (LPS) is een onderdeel van de buitenste membraan van Gram-negatieve bacteriën en speelt een belangrijke rol bij verschillende aandoeningen in de rundveesector. Vooral bij het kalf zijn LPS-gerelateerde ziektebeelden veel voorkomend, zoals neonatale diarree, pneumonie en septicemie. De experimentele toediening van LPS aan dieren werd reeds veelvuldig toegepast om de verschillende aspecten van de acute fase reactie te bestuderen. Dergelijke LPS inflammatiemodellen worden bovendien geadviseerd om de immunomodulerende eigenschappen van geneesmiddelen na te gaan. Immunomodulatie duidt in deze context op een relatief recent onderzoeksdomein waarin getracht wordt om de aangeboren immuunrespons zodanig te moduleren dat het herstel van het dier bevorderd wordt. Bijgevolg betreft het niet louter een immunosuppressief of anti-inflammatoir effect. Studies bij zowel de mens als laboratoriumdieren bevestigden reeds de immunomodulerende eigenschappen van bepaalde antimicrobiële geneesmiddelen (waaronder macrolide antibiotica), niet-steroïdale anti-inflammatoire geneesmiddelen (NSAIDs) en corticosteroïden, onafhankelijk van hun directe farmacodynamische effecten. Bij runderen daarentegen staat dit onderzoeksonderwerp nog in zijn kinderschoenen. Nochtans kan informatie over dergelijke eigenschappen van geneesmiddelen bijdragen tot de ontwikkeling van geoptimaliseerde behandelingsstrategieën voor LPS-geassocieerde aandoeningen, die op hun beurt voordelen hebben met betrekking tot productieresultaten en dierenwelzijn.

De **ALGEMENE DOELSTELLING** van dit doctoraatsonderzoek was daarom het bestuderen van de immunomodulerende eigenschappen van het macrolide gamithromycine (GAM), het corticosteroïd dexamethasone (DEX) en het NSAID ketoprofen (KETO), alsook hun combinaties (GAM-DEX en GAM-KETO), in een gestandaardiseerd LPS inflammatiemodel bij het kalf. De nadruk lag hierbij op de acute fase reactie.

In de **ALGEMENE INLEIDING** werd een overzicht gegeven van de beschreven *in vivo* LPS inflammatiemodellen bij het rund. Hierbij lag de focus op de LPS-geïnduceerde acute fase respons, met vrijstelling van pro-inflammatoire cytokines (tumor necrosis factor (TNF)-α, interleukine (IL)-1β en IL-6) en acute fase eiwitten (serum amyloid A (SAA) en haptoglobine (Hp)) en het optreden van volgende symptomen: acuut longfalen, koorts, tachycardie en gedragsveranderingen zoals depressie en anorexie. Daarnaast werden de resultaten van

boviene *in vitro*, *ex vivo* en *in vivo* studies met betrekking tot de immunomodulerende eigenschappen van antibiotica, NSAIDs en corticosteroïden besproken.

In **HOOFDSTUK 1** werd de ontwikkeling en karakterisatie van een LPS inflammatiemodel bij 4-weken-oude kalveren beschreven. Bij deze dieren werd een acute fase respons geïnduceerd via een intraveneuze toediening van ultra-zuiver *Escherichia coli* LPS aan een dosis van 0.5 µg/kg lichaamsgewicht. Deze challenge gaf aanleiding tot volgende symptomen: tachypnee (gemiddeld 20 min post challenge (p.c.)), decubitus (30 min p.c.), algemene depressie (1.75 h p.c.), koorts (5 h p.c.) en tachycardie (5 h p.c.). Na het normaliseren van de ademhalingssymptomen volgde een periode van algemene depressie, waarbij de depressie toenam met stijgende lichaamstemperatuur. Eenmaal de koortspiek voorbij was, werden de dieren geleidelijk aan terug alert en nam hun eetlust toe. Een volledig herstel werd na gemiddeld 6 h bereikt. Maximale plasmaconcentraties van de inflammatoire mediatoren TNF-α, IL-6, SAA en Hp werden gedetecteerd na 1, 3.5, 24 en 18 h, respectievelijk.

In **HOOFDSTUK 2** werden de farmacokinetische eigenschappen van KETO bepaald na intramusculaire (IM) toediening aan kalveren. Aangezien enantioselectieve dispositiekinetiek en chirale inversie beschreven werden voor dit NSAID werden de plasmaconcentraties van R(–) en S(+) KETO afzonderlijk gekwantificeerd. De resultaten toonden duidelijk hogere concentraties van het S(+) enantiomeer aan, alsook significant verschillende farmacokinetische parameters tussen R(–) en S(+) KETO. Het tijdstip waarop maximale concentraties (t_{max}) van het S(+) enantiomeer – de farmacologisch actieve vorm – bereikt werden, was 0.98 ± 0.18 h.

In **HOOFDSTUK 3** werden de immunomodulerende eigenschappen van GAM, DEX en KETO, en hun respectievelijke combinaties bestudeerd in ons gestandaardiseerde LPS inflammatiemodel. DEX werd hierbij gebruikt als positieve controle. Gebaseerd op studies van andere onderzoeksgroepen kon besloten worden dat voorbehandeling nodig was om een maximaal effect op de immuunrespons uit te oefenen. Om deze reden werden alle geneesmiddelen 1 h voor de LPS challenge toegediend, overeenkomstig met de t_{max} bij kalveren. De toediening van GAM gebeurde subcutaan, voor DEX en KETO was dit IM. Twee studies met een identiek proefopzet werden uitgevoerd (**HOOFDSTUK 3.1** en **HOOFDSTUK 3.2**),

hoewel Hp enkel in Hoofdstuk 3.1 bepaald werd, en prostaglandine E_2 (PGE_2) enkel in Hoofdstuk 3.2.

Niettegenstaande de resultaten van eerdere studies met macroliden had GAM geen invloed op de productie van TNF-α, IL-6, SAA, Hp en PGE_2, noch op de klinische symptomen na LPS. In tegenstelling tot GAM, inhibeerde DEX de vrijstelling van cytokines en resulteerde het in een sneller herstel van de dieren. De ademhalingssymptomen en de koortsontwikkeling werden daarentegen niet beïnvloed door het corticosteroïd. KETO vertoonde de meest uitgesproken effecten in het inflammatiemodel, aangezien de kalveren gezond bleven gedurende het experiment: het optreden van koorts, depressie en anorexie werd volledig onderdrukt. Bovendien lagen de cytokine en SAA concentraties lager na voorbehandeling met KETO, en was PGE_2 niet detecteerbaar. In de combinatiegroepen werden geen synergistische of additieve effecten vastgesteld. In de GAM-KETO groep daarentegen werden zelfs hogere cytokineconcentraties teruggevonden, hoewel deze geen invloed hadden op het klinische beeld.

Samengevat kan gesteld worden dat we een gestandaardiseerd en reproduceerbeer LPS inflammatiemodel ontwikkeld hebben bij kalveren voor de studie van de immunomodulerende eigenschappen van geneesmiddelen. KETO inhibeerde alle bestudeerde aspecten van de acute fase reactie: cytokine, SAA en PGE_2 concentraties, koorts, depressie en anorexie. GAM daarentegen had geen invloed, terwijl DEX een intermediair effect uitoefende. Deze bevindingen suggereren dat prostaglandines een belangrijkere rol spelen bij het induceren van LPS-gerelateerde ziektesymptomen in vergelijking met pro-inflammatoire cytokines. Bijgevolg beklemtoont dit doctoraatsonderzoek de voordelen van het gecombineerd gebruik van een antimicrobieel geneesmiddel en een NSAID voor de behandeling van acute bacteriële infecties bij runderen.

CURRICULUM VITAE

Elke Plessers werd geboren op 5 december 1984 te Bree. Na het behalen van haar diploma secundair onderwijs aan het Sint-Augustinusinstituut te Bree (Wiskunde-Wetenschappen), begon zij in 2002 met de studie Diergeneeskunde aan de Universiteit Gent. Hier studeerde ze in 2008 af met onderscheiding. Haar thesis handelde over morbiditeit en mortaliteit op vleeskalverbedrijven.

Na een korte periode in de rundveepraktijk startte ze in september 2009 als assistent aan de vakgroep Farmacologie, Toxicologie en Biochemie van de Faculteit Diergeneeskunde. Hier werkte ze aan een doctoraatsonderzoek over de immunomodulerende eigenschappen van geneesmiddelen in een lipopolysaccharide-inflammatie model bij het kalf, met Prof. dr. S. Croubels en Prof. dr. P. De Backer als promotoren. Naast haar eigen onderzoek stond ze mee in voor de toxicologische dienstverlening van de vakgroep, gaf ze practica farmacologie en begeleidde ze meerdere studenten in het behalen van hun Masterproef. In 2015 vervolledigde ze het trainingsprogramma van de Doctoral School of Life Sciences and Medicine van de Universiteit Gent.

Elke Plessers is auteur en mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Daarnaast was ze spreker op meerdere (inter)nationale congressen.

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